STUDIES ON BACTERIAL AMINO ACID RACEMASES AND THE GENE CLONING(Dissertation_全文)

AUTHOR(S):

Inagaki, Kenji

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CONTENTS

ABBREVIATIONS

CD	Circular dichroism
DEAE-Toyopearl	Diethylaminoethyl-Toyopearl
DNase I	Deoxyribonuclease I
EDTA	Ethylenediaminetetraacetic acid
HPLC	High performance liquid chromatography
Km	Michaelis constant
MBTH	3-Methyl-2-benzothiazolone hydrazone
MTCC	S-(N-Methylthiocarbamoyl)-L-cysteine
NAD	Nicotineamide adenine dinucleotide
NADH	Nicotineamide adenine dinucleotide reduced form
pyridoxal-P (PLP)	Pyridoxal 5'-phosphate
RNase A	Ribonuclease A
SDS	Sodium dodecyl sulfate
Tris	Tris(hydroxymethyl)aminomethane

INTRODUCTION

Although free forms of D-amino acids rarely occur in nature, the D-amino acids are important constituents of biological materials. Especially, D-alanine and D-glutamate are known as components of the peptidoglycan layer of cell walls in many bacterial species (Steenson et al., 1968). In addition, a variety of D-amino acids have been found in peptide antibiotics such as polymyxins, tyrocidins, bacitracins and gramicidins (Craig et al., 1952). Since the L-isomers of amino acids are generally produced in biosynthetic pathways, formation of D-isomers can arise either from equilibration of configuration at the α-carbon of the L-enantiomer (racemase action) or from appropriately chiral reduction of an imino acid (transaminase action). For example, in Bacillus sphaericus and Bacillus subtilis D-amino acid transaminase converts α-ketoglutarate to D-glutamate (Martinez-Carrion & Jenkins, 1965). This may be the source of D-glutamate incorporated into the cell wall of these organisms. On the other hand, D-alanine appears to be formed directly from L-alanine by the action of alanine racemase in many species of bacteria (Adams, 1976).

Soda & Osumi (1969) discovered an amino acid racemase with very low substrate specificity and purified the enzyme to homogeneity. During the course of studying the properties of this enzyme, I have found the occurrence of another new amino acid racemase which catalyzes the racemization of various amino acids including methionine and

cysteine in a cell-free extract of a bacterium isolated from soil. I here describe the purification and characterization of this new amino acid racemase with broad substrate specificity, with particular emphasis on the comparison to the enzyme from Ps. putida.

The amino acid racemases, at present known only as enzymes of bacteria, have not been characterized well enzymologically. In particular, alanine racemase, probably the most widely distributed enzyme in the group, has been purified to homogeneity only recently, because of difficulties of purification. The requirement of cofactor, has remained obscure; the homogeneous enzyme purified from Pseudomonas putida requires pyridoxal-P as the sole cofactor (Adams et al., 1974), whereas those from Bacillus subtilis (Diven et al., 1964), Pseudomonas sp. 3550 (Free et al., 1967), Staphylococcus aureus (Rose & Strominger, 1966), and Escherichia coli (Lambert, 1972) are unclear in cofactor requirement.

Wasserman et al. (1983) have recently found that there are at least two genes encoding for alanine racemases in Salmonella typhimurium. The enzyme encoded by the dadB gene that is essential for utilization of L-alanine as a source of carbon, energy, and nitrogen through the D-alanine dehydrogenase reaction has been purified from Escherichia coli cells containing the plasmid pSW12, which carries the cloned dadB gene (Wasserman et al., 1984). The physical and kinetic characterization of the racemase as well as its primary structure predicted by DNA sequencing was also reported (Wasserman et al.,

1984). The Salmonella dal gene-encoded alanine racemase (N. Esaki & C. T. Walsh, unpublished results) probably functions biosynthetically in the bacterial cell wall assembly.

Because of the pivotal role of alanine racemase in cell wall biosynthesis and its unique distribution to prokaryotes, the enzyme has been recognized as a target for antibacterial drugs. In fact, some halogenated derivatives of D-alanine and phosphonoalanine-containing dipeptides were found to act as antibacterials by blocking the racemization of L- to D-alanine (Kollonitsch et al., 1973; Manning et al., 1974; Allen et al., 1978; Atherton et al., 1979). Detailed studies of the reaction mechanism of alanine racemase and the chemistry and geometry of its active site are needed to develop racemase-directed antibacterials with more selective toxicity.

The use of thermostable enzymes from thermophilic microorganisms (Brock, 1967, 1970; Friedman, 1968) is of great advantage for enzyme industry because of the high stability at elevated temperature, at high and low pH, and even in organic solvent (Singleton, 1973). The thermostable enzymes are also advantageous for the use in enzyme sensors, clinical analyses and immobilized systems, although the mechanism of thermostability has not been fully elucidated. However, none of alanine racemases so far reported is an enzyme from thermophilic bacteria.

In this thesis, I describe cloning and expression of the alanine racemase gene from a thermophilic gram positive bacterium, Bacillus

stearothermophilus, in E. coli, rapid and simple purification of the thermostable enzyme, and its enzymatic characterization.

The comparative study of these two amino acid racemases with distinct substrate specificity would be of great value to elucidate the reaction mechanism and active site structures of amino acid racemases and also to shed light on the evolutionary aspect of these enzymes.

CHAPTER I

BROAD SUBSTRATE SPECIFICITY AMINO ACID RACEMASE OF *AEROMONAS CAVIAE*

I have found a new amino acid racemase catalyzing the racemization of various amino acids in *Aeromonas caviae* (= *Aeromonas punctata* subsp. *caviae*). In this chapter, the intracellular localization, the purification, and some of the properties of the enzyme are described.

EXPERIMENTAL PROCEDURES

Materials. DEAE-Toyopearl was purchased from Toyo Soda Kogyo, Tokyo, Japan; DEAE-sephadex A-50 and Sephadex G-200 were from Pharmacia Fine Chemicals, Uppsala, Sweden. Egg white lysozyme, DNase II, RNase A and the standard proteins for molecular weight determinations were obtained from Sigma. L-MTCC was provided by T. Kimura in this laboratory. The other chemicals were analytical-grade reagents, and obtained from Nakarai Chemicals, Kyoto, Japan.

Preparation of Membrane and Cytoplasmic fractions. The membrane and the cytoplasmic fractions were prepared as described Gorden *et al.* (1972). The cells were suspended in 10 mM potassium phosphate buffer

(pH 8.0), and 10 mM EDTA (disodium salt) and 0.5 mg/ml of egg white lysozyme were added to the solution. The suspension was incubated at 25°C for 1 h, and then repeatedly frozen (by liquid N_2) and thawed at 50°C to lyse the cells. To the viscous solution were added 20 mM $MgCl_2$, 100 μg/ml of DNase I and 100 μg/ml of RNase A and the mixture was incubated at 25° C for 1 h. The suspension was centrifuged at 30,000 rpm for 30 min. The pellet used as a membrane fraction in this experiment was resuspended and washed by centrifugation in a small volume of potassium phosphate buffer (pH 8.0). The supernatant solution was employed as a cytoplasmic fraction.

Microorganism and Conditions of Culture. The isolated bacterium was identified as Aeromonas caviae (= A. punctata subsp. caviae) by American Type Culture Collection. The basal medium for cultivation consisted of 10 g of polypepton (Daigo, Japan), 10 g of meat extract (Kyokuto, Japan) and 5 g of NaCl per one liter of tap water. The pH of the medium was adjusted to 7.0. The large-scale cultivation was carried out at 30 °C for 16 h under aeration initially in 22-l of the medium placed in a 30-l Marubishi fermentor jar, and transferred into 250-l NBS fermentor jar containing 200-l of the medium. The cells harvested by centrifugation was washed with 0.85% NaCl solution, and then with 10 mM potassium phosphate buffer (pH 8.0) containing 10 μM pyridoxal-P and 0.01% 2-mercaptoethanol. The yield of wet cells was about 8.7 g/liter of the medium, and the washed cells were frozen at -20° C until use.

Enzyme Assay and Analytical Methods. The enzyme activity was determined at 25°C by measuring the change in optical rotation at 365 nm with a Perkin-Elmer 241 polarimeter. The standard assay mixture consisted of 20 μmol of potassium phosphate buffer (pH 8.0), 100 μmol of L-lysine, and enzyme in a final volume of 2.0 ml. Protein was determined by the Buiret method (Gonall et al., 1949) with bovine serum albumin as a standard. The concentration of the purified enzyme was determined by the use of the absorbance coefficient ($A^{1\%}_{280nm}$ =10.4), obtained by the method of Perlman & Longworth (1948). One unit of enzyme is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of L-amino acid into D-amino acid per min. Specific activity is expressed as units per mg of protein.

Time dependent inactivation of the enzyme by L-MTCC was measured at 25°C in 80 mM potassium phosphate buffer (pH 7.5) containing 50 μM pyridoxal-P in a final volume of 80 μl. Aliquats (20 μl) were removed at intervals and diluted 100 times with a standard assay mixture to determine the remaining activity. For studies of protective effect of amino acid on the inactivation by MTCC, an appropriate concentration of amino acid was added to the incubation mixture prior to the addition of the enzyme.

Spectrophotometric measurements were made with a Shimadzu UV3000 recording spectrophotometer with a 1.0-cm light path.

Enantiomers of amino acids were separated by the reversed-phase liquid chromatography using a JASCO Trirotar HPLC with a Finepack

(C_{18}) column (4.6 X 250 mm) in 8 mM copper acetate containing 17 mM L-proline, pH 5.0, according to Gil-Av et al. (1980). The amino acids eluted were determined with o-phthalaldehyde and 2-mercaptoethanol (Benson & Hare, 1975). The mobile phase and the o-phthalaldehyde reagent were mixed at a flow rate of 0.5 and 0.6 ml/min, respectively.

Polyacrylamide Gel Electrophoresis. Analytical electrophoresis in polyacrylamide gels was carried out in Tris/glycine buffer (pH 8.3) according to the method of Davis (1964). Gels were stained for protein with coomassie brilliant blue G-250 and destained in 7% acetic acid. SDS gel electrophoresis was performed in 10% of the polyacrylamide gels using the Tris/glycine buffer system described by King & Laemmli (1971). Relative molecular mass of subunit of the enzyme was determined from the relative mobility of standard proteins.

Ultracentrifugal Analysis. The purity of the purified enzyme and its sedimentation coefficient were determined with a Spinco Model E ultracentrifuge equipped with a phase plate as a schlieren diaphragm. The molecular weight of the enzyme was determined by the ultracentrifugal sedimentation equilibrium method according to the procedure of Van Holde & Baldwin (1958). The experiments were carried out in a Spinco Model E ultracentrifuge equipped with Raileigh interference optics. Multicell operations were employed in order to perform the experiment on four samples of different initial concentrations ranging from 0.9 to 3.8 mg/ml with the use of An-G rotor and double cells of different side-wedge angles. The rotor was centrifuged at 8,225 rpm

for 20 h at 20°C. Interference patterns were photographed at intervals of 30 min to compare and make sure that the equilibrium was established. The relation between the concentration of the enzyme and the fringe schift was determined using the synthetic boundary cell.

Amino Acid Analysis. Amino acid analysis was performed according to the method of Spackman et al. (1958) with a Hitachi 835 high performance amino acid analyzer. The enzyme was hydralyzed in 6N HCl at 110°C under reduced pressure for 24, 48, and 72 h. The hydrolysates were evaporated to dryness and subjected to amino acid analysis in duplicate. Half-cystine was determined as cysteic acid after performic acid oxidation and hydrolysis. Tryptophan and tyrosine were determined spectrophotometrically by the method of Edelhoch (1967).

Preparation of Antiserum. One milliliter of 0.5 mg pure enzyme in 10 mM potassium phosphate buffer (pH 7.2) was emulsified with 1 ml of Freund's complete adjuvant (Difco) and injected into a multiple subcutaneous site on the back of a young, male rabbit. After 4 weeks, the animal received a booster injection subcutaneously on the neck with 0.5 mg of the antigen homogenized in an equal volume of the complete adjuvant. On the 7th and 14th day after the booster injection, blood was collected from the ear vein and allowed to clot. The serum was centifuged at 6,000 X g for 10 min and stored at -20°C. Ouchterlony plates were made (Ouchterlony, 1953) using a 1% special agarose in 10 mM potassium phosphate buffer (pH 7.2) containing 0.85% NaCl. About 4 mm diameter wells were punched in a pattern of wells

with center-to-center distances of 15 mm. After filling the wells with appropriate proteins, the plates were allowed to stand at 30°C in a humid atomosphere for 1 day.

RESULTS

Intracellular Localization of Amino Acid Racemase. A. caviae cells cultivated at 30°C for 16 h were treated with lysozyme, and the cell lysate was separated into cytoplasmic and membrane fractions by ultracentrifugation (Scheme I). The resultant cytoplasmic fraction

Scheme I

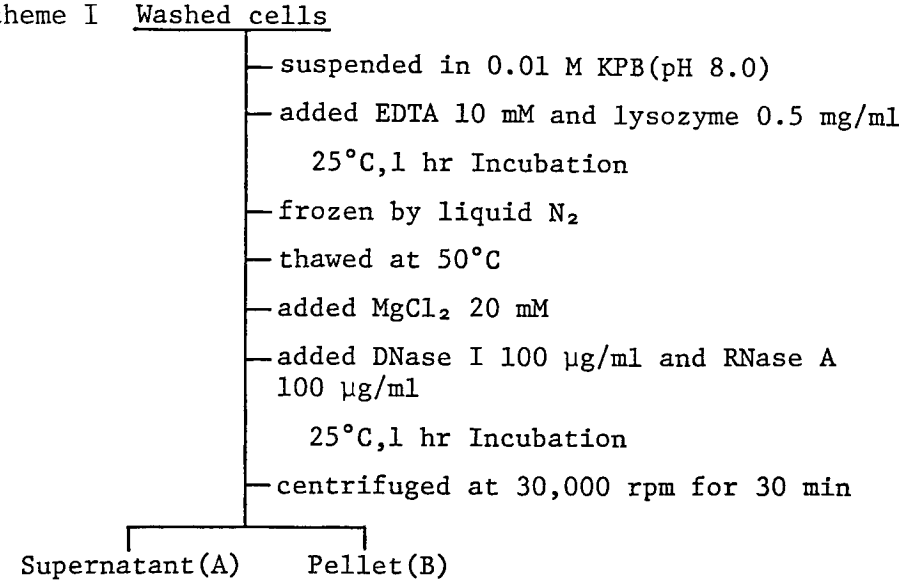


Table I Amino Acid Racemase Activity in Cytoplasmic and Membrane Fraction

	Specific activity (units/mg)	Total activity (units)
Cytoplasmic fraction(A)	2.0	141.2
Membrane fraction(B)	0.16	16.4

contained about 90% of the total amino acid racemase activity, whereas the membrane fraction had only less than 10% of the total activity (Table I). This result clearly indicates that the enzyme occurs in the cytoplasm, and is distinct from arginine racemase which has been reported to be a membrane-bound enzyme (Yorifuji, et al., 1971).

Purification of Enzyme. Unless otherwise stated, all operations were carried out at 0 - 5°C. Potassium phosphate buffer (10 mM, pH 8.0) containing 10 μM pyridoxal-P and 0.01% 2-mercaptoethanol was used as the standard buffer (Buffer A).

Step 1. The washed cells (800 g, wet weight) were suspended in 1 liter of Buffer A and disrupted continuously by a DYNO-MILL apparatus (Willey A. Bachofen Maschinenfabrik) with glass beads (0.1-0.2 mm in diameter). The supernatant solution obtained by centrifugation was used as the cell-free extract (1.2 liter).

Step 2. The cell-free extract was dialyzed against Buffer A and applied to a DEAE-Toyopearl column (8 X 50 cm). After the column was washed with 2 liters of Buffer A, the enzyme was eluted with the buffer containing 50 mM KCl. The active fractions were collected and concentrated by ultrafiltration followed by dialysis against Buffer A.

Step 3. The enzyme solution (65 ml) was placed on a hydroxyapatite column (2.5 X 20 cm) equilibrated with Buffer A. The column was washed with 200 ml of Buffer A and the enzyme was eluted with 15 mM potassium phosphate buffer (pH 8.0). The active fractions were pooled and concentrated.

Step 4. The enzyme solution (7.5 ml) was applied to a DEAE-Sephadex A-50 column (1.5 X 10 cm). After the column was washed with 60 ml of Buffer A and with 60 ml of Buffer A containing 20 mM KCl, the enzyme was eluted with Buffer A containing 50 mM KCl. The enzyme solution was concentrated.

Step 5. The enzyme (3.5 ml) was applied to a Sephadex G-200 column (3 X 130 cm) equilibrated with Buffer A, and eluted with the same buffer. The active fractions were pooled and concentrated.

Step 6. The enzyme (2.0 ml) was finally purified by chromato graphy on a hydroxyapatite column (0.6 X 4 cm) with Buffer A as an eluting buffer. The active fractions were combined and concentrated by ultrafiltration. Approximately 570-fold purification was achieved with an over-all yield of 40%. A summary of the purification procedure is presented in Table II.

Table II. Summary of Purification of Amino Acid Racemase. The activity was determined at 25°C by measuring the change in optical rotation at 365 nm. Protein was determined by the Buiret method (Gonall *et al.*, 1949).

Steps	Total Protein (mg)	Total Activity (10^4 U)	Specific Activity (U/mg)	Yield (%)
1. Crude extract	64,000	14	2.2	100
2. DEAE-Toyopearl	1,250	14	112	100
3. 1st Hydroxyapatite	200	14	700	100
4. DEAE-Sephadex A-50	100	9.4	938	67
5. Sephadex G-200	50	5.8	1,150	41
6. 2nd Hydroxyapatite	45	5.6	1,250	40

Purity and Molecular Weight. The purified enzyme was shown to be homogeneous by the criteria of disc gel electrophoresis (Figure 1A) and ultracentrifugation (Figure 1B). The sedimentation coefficient of the enzyme, calculated for water at 20°C, is 5.5S. The molecular weight of the enzyme was determined by the sedimentation equilibrium method. Assuming a partial specific volume of 0.74, a molecular weight of 76,000 was obtained. The molecular weight of the enzyme was also determined by Sephadex G-100 gel filtration method. On the basis of the plots of the reduced elution volumes (Ve/Vo) versus logarithm of molecular weight, the molecular weight of amino acid racemase was calculated to be about 73,000.

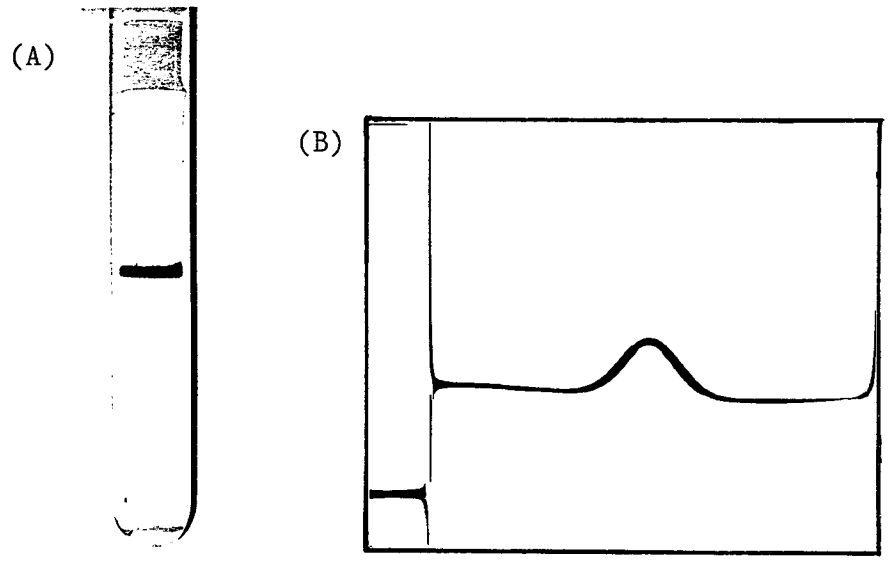


Figure 1: Disc gel electrophoresis (A) and sedimentation pattern (B) of amino acid racemase. (A) Purified Enzyme (20 µg) was subjected to electrophoresis under the conditions described (Davis, 1964). The direction of migration is from cathode (top) to anode. (B) Sedimentation pattern was obtained at 1.5 mg protein/ml in 10 mM potassium phosphate buffer (pH 7.2). The picture was taken at 72 min after achieving top speed (42040 rev./min).

Structure of Subunit. The subunit structure of the enzyme was examined by disc gel electrophoresis (King & Laemmli, 1971). The enzyme was incubated with 1.0% SDS in 10 mM sodium phosphate buffer (pH 7.0) containing 1% 2-mercaptoethanol at 37°C for 2 h. The treated enzyme preparations were subjected to electrophoresis in the presence of 0.1% SDS. There was a single band of stained protein (Figure 2). To determine the molecular weight of the polypeptide in this band, a series of marker proteins treated in the same manner was run. The molecular weight was calculated to be about 40,000 from a semilogarithmic plot of molecular weight against mobility (Figure 2). Thus, the enzyme consists of two subunits identical in molecular weight.

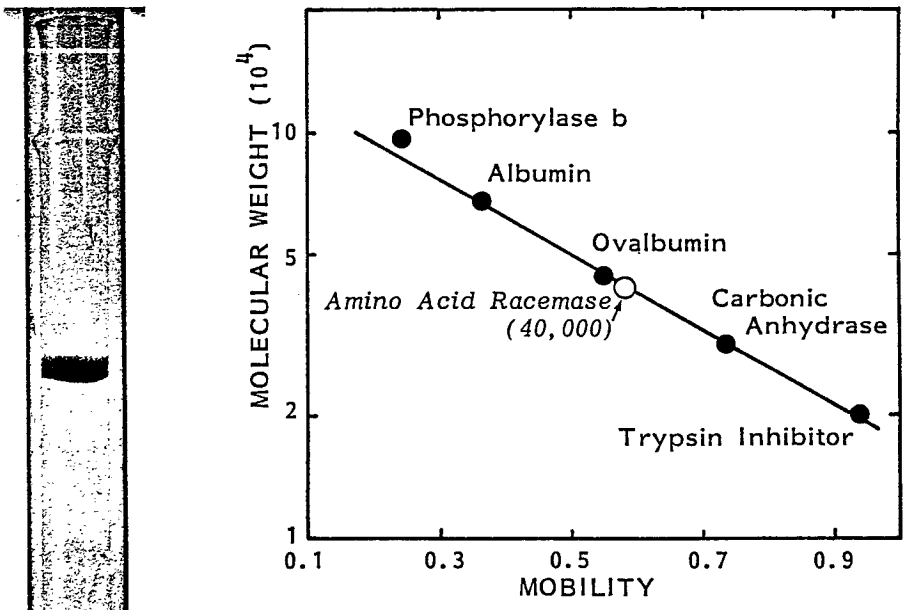


Figure 2: Determination of molecular weight of the amino acid racemase subunit by SDS gel electrophoresis. After the purified enzyme (20 μg) was treated with 1.0% SDS in 10 mM sodium phosphate buffer (pH 7.2) containing 1.0% 2-mercaptoethanol and 25% glycerol at 37°C overnight, the treated enzyme preparation was subjected to electrophoresis in the presence of 0.1% SDS with 10% polyacrylamide gels in the Tris-glycine buffer system.

Amino Acid Composition. The amino acid composition of the enzyme is given in Table III. The predominant residues of the enzyme protein were aspartic acid, glutamic acid, alanine, glycine, and leucine. The total of the integral numbers of each amino acid residues gave a calculated molecular weight of about 40,000 for the polypeptide chain.

Table III Amino Acid Composition of Amino Acid Racemase

Amino Acid	Number of Residues (mol/mol of Enzyme)			Number of Proposed Amino Acid Residues
	24 h	48 h	72 h	
Aspartic acid	38.0	37.9	38.3	38
Threonine	17.4	17.1	17.6	19
Serine	11.6	9.8	11.6	13
Glutamic acid	31.6	31.4	32.2	32
Proline	11.9	11.6	14.4	12
Glycine	30.2	29.4	30.0	30
Alanine	35.6	34.8	35.1	35
Half-cystine	0.1	0.0	0.2	0
Valine	21.9	23.4	22.6	23
Methionine	11.8	11.6	11.8	12
Isoleucine	18.9	19.9	19.8	20
Leucine	27.2	27.4	27.5	28
Tyrosine	12.1	12.4	13.0	13
Phenylalanine	7.3	7.0	7.1	7
Lysine	23.1	23.4	22.6	23
Histidine	7.1	7.2	7.4	7
Arginine	15.4	15.1	15.4	15
Tryptophan				4
Total Number of Residues				331

The protein was hydrolyzed at 110°C for 24, 48, and 72 h. The results were averaged and the integral number of the amino acid is presented. Details for the experiment are given in the text.

Characterization of Cofactor. The purified enzyme exhibited absorption maxima at 280 and 420 nm with an A_{280}/A_{420} ratio of 5.7 (Figure 3A). The absorption spectrum was not influenced substantially by varying pH (6.0-9.0). The occurrence of the absorption peak at 420 nm suggests that the enzyme contains pyridoxal-P as a cofactor and that the formyl group of the bound pyridoxal-P forms an azomethine linkage with an amino group (probably an ε-amino group of a lysine residue) of the enzyme protein, as in other pyridoxal-P enzymes studied so far. Reduction of the enzyme with sodium borohydride by the dialysis method of Matsuo & Greenberg (1959) affected both the activity and the absorption spectrum (Curve C in Figure 3A). The reduced enzyme was catalytically inactive and the addition of pyridoxal-P did not recover the activity. This result suggests that sodium borohydride reduces the azomethine linkage to bind the cofactor covalently. The amount of pyridoxal-P bound in the enzyme was examined by the phenylhydrazine method (Wada & Snell, 1961) and the MBTH method (Soda et al., 1969). An average pyridoxal-P content of 1 mol/40,000 g of protein was obtained. This shows that two mol of pyridoxal-P are bound to one mol of enzyme protein. The holoenzyme was converted to the apoenzyme by dialysis against 10 mM potassium phosphate buffer (pH 8.0) containing 10 mM hydroxylamine and 0.01% 2-mercaptoethanol for 24 h. The apoenzyme had neither the absorption maximum at 420 nm (Curve B in Figure 3A) nor the activity without addition of excess pyridoxal-P. The apparent dissociation constant for pyridoxal-P was determined to be

2.6 μM. The circular dichroic spectra of the holoenzyme (Figure 3B) was also measured at pH 8.0. The enzyme showed negative circular dichroic extremes at 280 and 420 nm, corresponding to the absorption maxima.

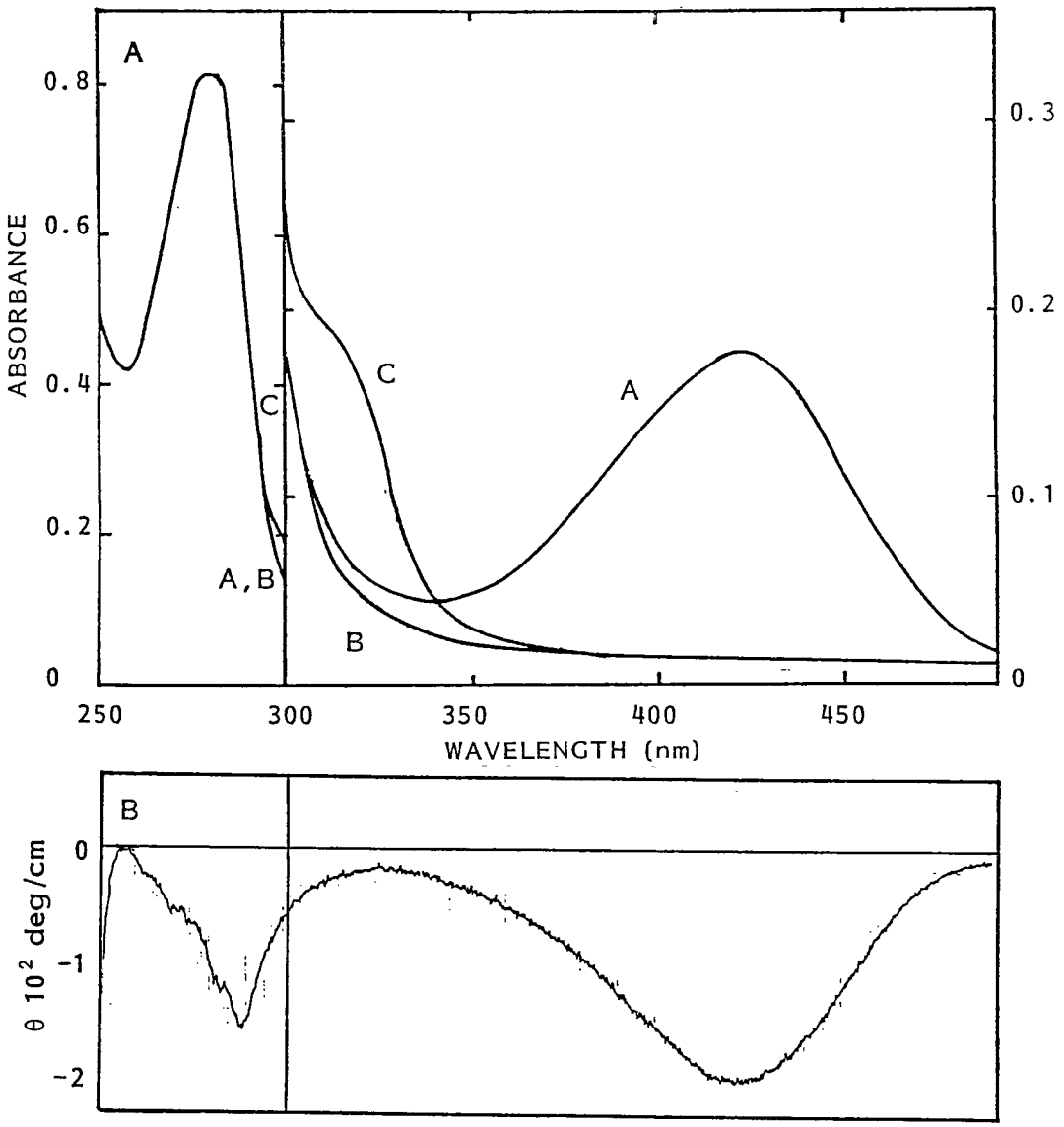


Figure 3: Absorption (A) and circular dichroic (B) spectra of amino acid racemase. A,the absorption spectra were taken in 10 mM potassium phosphate buffer (pH 8.0). Curve A, holo enzyme; Curve B, apoenzyme; Curve C, NaBH -reduced enzyme. B,the circular dichroic spectrum was in 10 mM potassium phosphate buffer (pH 8.0).

Identification of Reaction Product. The enzyme reaction product from D-methionine as a substrate was analyzed by the high performance reversed-phase chromatography. This method and the reaction conditions are described under "Experimental Procedures". As shown in Figure 4, a new peak whose retention time was 59 min under the conditions (the retention time of D-methionine = 28 min) and corresponded to that of L-methionine, appeared on the chromatogram after 30 min incubation with the enzyme. The peak areas of D- and L-methionine are almost equal, indicating that the enzymatic racemization of D-methionine completed within 30 min. Figure 5 also shows the enzymatic racemization of D-,L-lysine and D-,L-methionine monitored by polarimeter. The change of the optical rotation is initially very rapid and gradually becomes slow. After incubation for 20 min, the solution was subjected to an amino acid analyzer. Except for the substrate amino acid, no other amino acid and ammonia was detected.

Substrate Specificity and Kinetics. The substrate specificity of the enzyme was compared with that of amino acid racemase from Pseudomonas putida and of arginine racemase from Pseudomonas taetrolens in Table IV. The amino acid racemase of A. caviae has very broad substrate specificity as well as the enzyme of Ps. putida. In addition to lysine, which is the most preferred substrate, various other amino acids serve as a substrate for the enzyme. In particular, basic amino acids, sulfur containing amino acids, and glutamine are good substrates. Acidic amino acids and aromatic amino acids are inert.

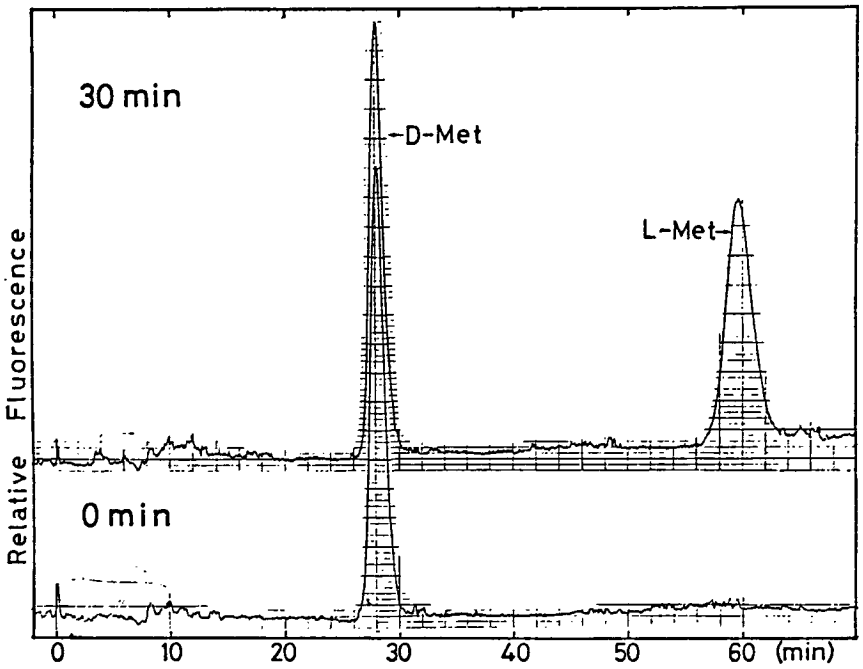


Figure 4: Identification of the reaction product by reversed-phase chromatography. Method and the reaction conditions are described in "Experimental Procedures".

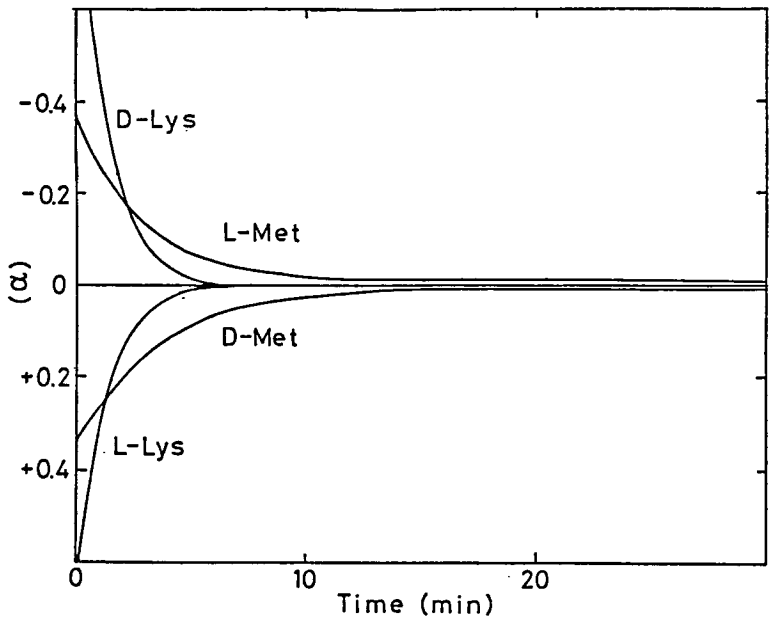


Figure 5: Enzymatic racemization of amino acids. Reaction conditions; 50 mM potassium phosphate buffer (pH 8.0), 50 mM D- or L- amino acid and the enzyme. Optical rotation was measured with a Perkin-Elmer 241 polarimeter.

Table IV. Substrate Specificity of Amino Acid Racemase

Substrates	Relative activity		
	Enzyme of *A.punctata*	Enzyme of *Ps.putida*	Enzyme of *Ps.taetrolens*
L-Lysine	100	100	100
L-Ornithine	79	61	40
L-Ethionine	67	76	12
L-Arginine	65	60	91
L-Glutamine	57	24	6
L-Homocysteine	55	29	-
L-Methionine	45	48	4
S-CH_3-L-Cysteine	43	-	-
ε-N-Acetyl-L-Lysine	37	40	78
L-Homocitrulline	34	-	11
L-Citrulline	30	17	12
L-Homoarginine	29	-	23
L-Norleucine	28	21	-
L-SeHomocysteine	27	38	-
L-Leucine	12	3	0
L-Homoserine	12	10	0
L-Asparagine	9	2	0
L-Alanine	7	9	0
L-Serine	3	9	0
L-Cysteine	3	3	0
L-Threonine	2	0	0
L-Valine	0	0	0
L-Glutamic acid	0	0	0
L-Aspartic acid	0	0	0
L-Proline	0	0	0
L-Tyrosine	0	-	-
L-Tryptophane	0	-	-
L-Phenylalanin	0	-	-

The enzyme activity was determined by measuring the change in optical rotation at 365 nm or HPLC.

Apparent Michaelis constants for several substrates were determined by the Lineweaver-Burk plot; L-lysine, 1.0 mM; D-lysine, 0.9 mM; L-ornithine, 0.9 mM; L-arginine, 1.0 mM; and D-methionine, 2.5 mM.

Effect of pH. When the enzyme activity was examined in 0.2 M potassium phosphate, Tris-HCl, and Britton-Robinson's buffer (Britton & Robinson, 1931), the enzyme showed the maximum reactivity in the pH range of 7.5 9.0.

Effect of Temperature on Enzyme Activity and Stability. The effect of temperature on the amino acid racemase activity was performed at pH 8.0 (Figure 6). The maximum reaction velocity was obtained at 60 °C. The reaction rate increased linearly when the temperature was raised in the range of 30-60°C. However, the velocity decreased rapidly over 70°C. When the enzyme in 0.1 M KPB (pH 8.0) was heated at various temperatures for 10 min and 30 min, the enzyme was found stable up to 50°C (Figure 7).

Inhibitors. Various compounds were investigated for their inhibitory effects on enzyme activity. The enzyme was strongly inhibited after a 10 min incubation at 37 °C with hydroxylamine-HCl, L-penicillamine, D-penicillamine, and D-cycloserine, which are typical inhibitors for pyridoxal-P enzymes (inhibition at 1 mM: 85%, 30%, 50%, and 45%, respectively)(Table V). Enzyme activity was not inhibited by semicarbazide, p-chloromercuribenzoic acid (PCMB), monoiodoacetic acid, arsenite, iodoacetamide, N-ethylmaleimide (NEM), and EDTA.

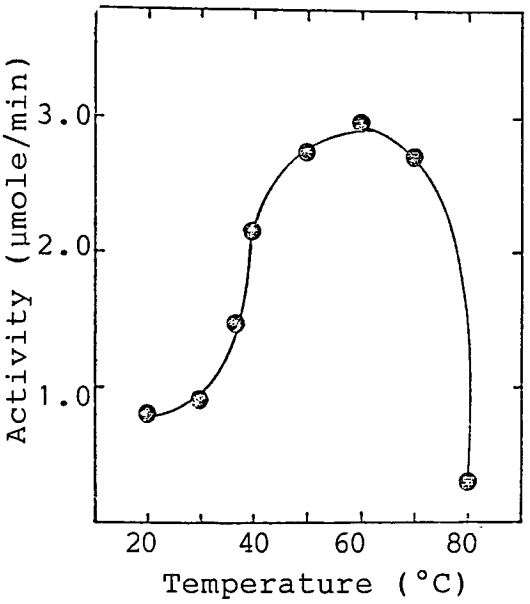


Figure 6: Optimum temperature of amino acid racemase. The enzyme activity was measured by polarimeter.

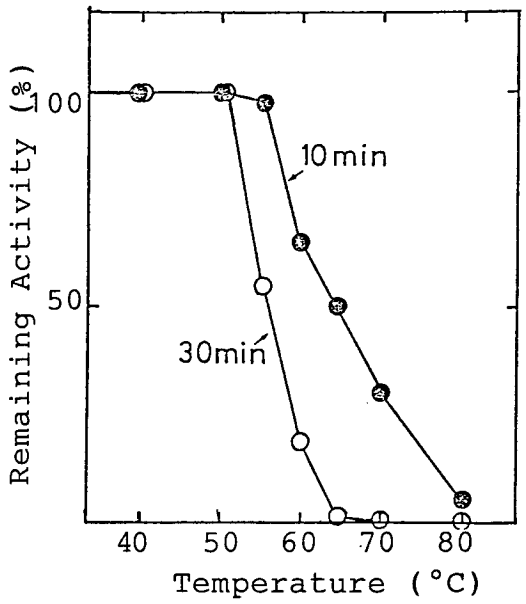


Figure 7: Heat stability of amino acid racemase. The enzyme was subjected to the heat treatment with 0.1 M potassium phosphate buffer (pH 8.0).

Table V. Effect of Inhibitors on the enzyme. The enzyme activity was measured under the standard conditions after the enzyme was pre-incubated for 10 min with inhibitors at a concentration of 1 mM.

Inhibitor	Conc. (mM)	Inhibition (%)
Hydroxylamine	1	85
L-Penicillamine	1	30
D-Penicillamine	1	50
D-Cycloserine	1	45
$HgCl_2$	1	50
Iodoacetamide	1	0
Monoiodoacetate	1	0
PCMB	1	0
N-Ethylmaleimide	1	0
$NaAsO_2$	1	0
EDTA	1	0

Inactivation of Amino Acid Racemase by MTCC. When the enzyme was preincubated with L-MTCC, the racemase activity for basic amino acids such as L-lysine, L-ornithine and L-arginine was lost faster than that for neutral ones such as L-methionine, L-glutamine and L-alanine. The protective effect of various substrate amino acids and related compounds on the inactivation of the enzyme by L-MTCC was examined. In addition to L-lysine, several other basic amino acids such as L-arginine and L-ornithine protected effectively the enzyme from the inactivation, whereas neutral amino acids such as L-alanine and L-methionine were much less effective (Figure 8).

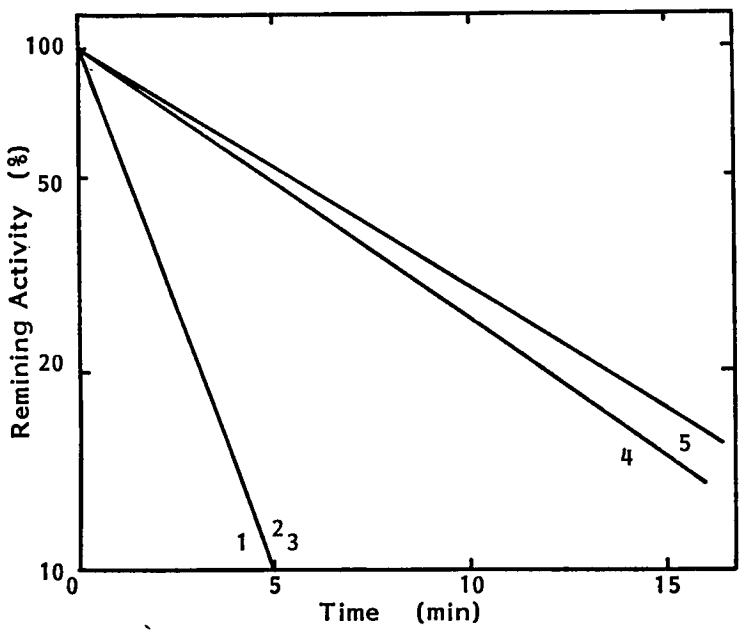
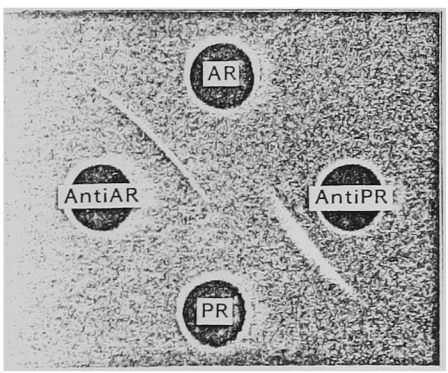


Figure 8: Protective effect of various amino acids on inactivation of the enzyme by L-MTCC. Amino acid racemase (10 μg) was added to a solution containing L-MTCC (10 mM) and an amino acid (50 mM). After incubation at 25°C, remaining racemase activity for L-lysine was measured as described in the text. 1, none; 2, alanine; 3, methionine; 4, ornithine; 5, lysine.

Ouchterlony Double-Diffusion Analysis. Antisera against the purified amino acid racemases of Pseudomonas putida and Aeromonas caviae were prepared in order to investigate their immunochemical relationship. The result of Ouchterlony double-diffusion analysis is presented in Figure 9. The antiserum against the A. caviae enzyme did not form a precipitin band with the Ps. putida enzyme, but formed it only with the A. caviae enzyme. Similarly, the antiserum against the Ps. taetrolens enzyme reacted only with the Ps. taetrolens racemase (data not shown). Thus, it is evident that the racemase of A. caviae is immunochemically distinct from the amino acid racemase of Ps. putida and the arginine racemase of Ps. taetrolens.

Figure 9: Ouchterlony double-diffusion analysis of amino acid racemase.



AR : Amino Acid Racemase of *Aeromonas punctata*

PR : Amino Acid Racemase of *Pseudomonas putida*

AntiAR Antiserum against AR

AntiPR : Antiserum against PR

DISCUSSION

Amino acid racemase has been purified to homogeneity from cell-free extracts of the isolated mesophilic bacterium Aeromonas caviae. Enzymological and physicochemical properties of this enzyme were compared with those of the enzyme from Ps. putida (Soda & Osumi, 1969,1971) and Ps. taetrolens (Yorifuji et al., 1971) in Table VI. Among three racemases, arginine racemase of Ps. taetrolens is clearly distinct from other two enzymes. Arginine racemase is composed of four identical subunits and contains four pyridoxal-P. Yorifuji et al. (1971) indicated that arginine racemase exsists in the membrane fraction. Although the amino acid racemase of A. caviae is immunochemically distinct from the amino acid racemase of Ps. putida, these two racemases are very similar in many aspects, particularly in physicochemical properties such as molecular weight and subunit structure. Enzymological properties such as optimum pH and substrate specificity are also similar. Kimura et al. (1985) indicated that amino acid racemase of Ps. putida has two binding sites in its active center: one of them binds the α-amino and carboxyl groups of both neutral and basic amino acids (Site-A), where the racemization (removal of α-proton of a substrate and reprotonation) occurs, and the other site binds the ω-amino group of basic amino acids (Site-B). This suggestion is in good agreement with a fact that basic amino acids are the preferrable substrates for these broad substrate specificity amino

acid racemases. Amino acid racemase of A. caviae is inactivated by L-MTCC as well as the enzyme of Ps. putida. Protective effect of amino acids on inactivation by L-MTCC is also very similar to that for Ps. putida enzyme. These results suggest the homologous structure of these enzymes' active sites.

Ps. putida is a strictly aerobic bacterium, whereas A. caviae is a facultatively anaerobic bacterium. Thus, the similality of these enzyme is of interest, suggesting that the enzymes are evolutionally related. In contrast with most of amino acid racemases such as alanine racemase, these two enzymes have a very broad substrate specificity (see Table IV). I have found that cysteine, homocysteine, Se-cysteine, and Se-homocysteine are substrates for these broad substrate specificity amino acid racemase. Methionine racemase was discovered by Shockman & Toennis (1953) and partially purified by Kallio & Larson (1955). Lysine racemase was discovered by Huang & Kita (1958) and partially purified by Ichihara et al. (1960). These studies suggested methionine racemase and lysine racemase were pyridoxal-P enzymes, but their substrate specificity and other enzymological properties have remained unsettled. It is likely that these enzymes are identical with or similar to the broad substrate specificity amino acid racemase described in this thesis.

Among various amino acid racemases thus far studied, alanine racemase of Escherichia coli and arginine racemase of Ps. taetrolens are the only enzyme whose intracellular localization has been demonst-

rated. Alanine racemase is the initial enzyme of the alanine branch in the biosynthetic route of uridine diphosphate (UDP)-N-acethylmuramyl-pentapeptide, a precursor of the cell wall peptidoglycan (Neuhaus, 1967). Kaczorowski et al. (1975) demonstrated that alanine racemase of E. coli participates in the active transport system. These findings were supported by the fact that alanine racemase was localized in the membrane. I have shown that amino acid racemase of A. caviae is localized in the cytoplasm. The physiological function of the enzyme is unknown at present.

Table VI. Properties of Amino Acid Racemases

Properties		Amino Acid Racemase of		
		A. punctata	*Ps. putida*	*Ps. taetrolens*
Mol. Wt.	:	80,000	84,000	167,000
S_{20w}	:	5.5 S	5.6 S	—
λ_{max} (ε_M)	:	280 nm (87,200) 420 nm (14,600)	280 nm (93,200) 420 nm (18,600)	280 nm (155,300) 420 nm (31,100)
$E^{1\%}_{280}$	:	10.9	8.5	9.3
Subunit (Mol. Wt.)	:	2 identical (40,000)	2 identical (42,000)	4 identical (42,000)
Cofactor	:	PLP 2 mol/mol Enz	PLP 2 mol/mol Enz	PLP 4 mol/mol Enz
Optimum Temp.	:	60°C	45°C	—
Optimum pH	:	Lys 7.5 - 9.0 Orn 8.0 - 8.5	Lys 7.5 - 9.0 Arg 10.0 - 11.0	Lys 7.5 - 10.6 Arg 9.0 - 10.6
Km	:	PLP 2.6 μM D-Lys 0.2 mM D-Met 2.5 mM	PLP 24 μM D-Lys 0.1 mM D-Met 2.7 mM	PLP 0.4 μM D-Arg 1.0 mM
Localization	:	Cytosol	Cytosol	Membrane

SUMMARY

An amino acid racemase, which occurs in the cytoplasmic fraction of Aeromonas caviae, has been purified to homogeneity by the criteria of electrophoresis and ultracentifugation.

The enzyme has a molecular weight of about 80,000 and consists of two subunits identical in molecular weight (about 40,000). The enzyme contains 2 mol of pyridoxal-P per mol of enzyme, and exhibits absorption maxima at 280 nm and 420 nm. The holoenzyme is resolved by dialysis against hydroxylamine to yield the inactive apoenzyme, which is reconstituted by the addition of pyridoxal-P to recover the full activity. The enzyme catalyzes racemization of a number of amino acids, e.g. lysine, ornithine, ethionine, arginine, glutamine, and methionine. The Michaelis constants were determined: 1 mM for L-lysine; 0.9 mM for D-lysine; 0.9 mM for L-ornitnine; 1 mM for L-arginine; and 2.6 μM for pyridoxal-P.

This enzyme is similar in enzymological properties to the racemase of Pseudomonas putida, but is distinct from it in immunochemical properties.

CHAPTER II

THERMOSTABLE ALANINE RACEMASE OF *BACILLUS STEAROTHERMOPHILUS*

Although alanine racemases have been purified from several bacteria (see Adams, 1976), few have been investigated in detail. In this chapter, I describe the cloning and expression of an alanine racemase gene from a thermophilic gram positive bacterium, *Bacillus stearothermophilus*, in *E. coli*, rapid and simple purification of the thermostable enzyme to compare its properties with the broad substrate specificity amino acid racemase descrived in the previous chapter.

EXPERIMENTAL PROCEDURES

Materials. Egg white lysozyme, RNase A and chloramphenicol were obtained from Sigma Chemicals Co. The endonucleases (*Eco*RI, *Hind*III, *Sal*I, and *Pst*I), T4 DNA ligase and bacteriophage λ DNA were purchased from Takara Shuzo Co. (Kyoto, Japan). Pronase P (Kaken Kagaku Co.), and dithiothreitol and ATP (Nakarai Chemicals Co.) were also the commercial products. The other chemicals were of highest purity available.

Strains and Media. Bacillus stearothermophilus IFO 12550 and Escherichia coli C600 $r_k^- m_k^-$ thi thr leu were used as a donor strain of the gene and the host strain for plasmid constructions, respectively. Transformed E. coli cells were grown in L broth (1% polypeptone, 0.5% yeast extract, 0.5% NaCl and 0.1% glucose; pH 7.2) supplemented with 2% agar and appropriate antibiotics. Antibiotic concentrations used for the selection of transformants were 25 μg/ml of ampicillin and 15 μg/ml of tetracycline. B. stearothermophilus was grown at 55°C in a Medium A (pH 7.2) containing 1.5% polypeptone, 0.1% glycerol, 0.01% yeast extract, 0.01% meat extract, 0.5% NaCl, 0.1% KH_2PO_4, 0.2% K_2HPO and 0.01% $MgSO_4 \cdot 7H_2O$ with shaking.

Enzyme and Protein Assays. Enzyme assays were performed at 50°C. A unit of enzyme is that amount which catalyzes the formation of 1 μmol of product per min at pH 9.0. The specific activity is expressed as units per mg of protein. Activity was mesured in the D- to L- alanine direction by monitoring the production of NADH at 340 nm on a Union Giken SM401 recording spectrophotometer as the L-alanine was converted to pyruvate and ammonia by L-alanine dehydrogenase. A saturating amount of L-alanine dehydrogenase (ca. 50 units) was added to the assay mixture during the course of racemase purification, but the addition was not necessary when assaying the racemase activity in transformants which carried a plasmid encoding the L-alanine dehydrogenase gene. A standard assay contained 100 μmol of D-alanine, 100 μmol of Glycine-KOH buffer, 2.5 μmol of NAD, 50 units of L-alanine

dehydrogenase, and alanine racemase at pH 9.0 in a 1 ml volume. L-Alanine dehydrogenase obtained from B. stearothermophilus (about 20% pure) was provided by Y. Sakamoto in this laboratory. Racemase activity was assayed also by measuring the change in optical rotation at 365 nm with a Perkin-Elmer 241 polarimeter. A photocell with a 10-cm light path contained 100 μmol of D-alanine, 250 μmol of potassium phosphate buffer (pH 8.0) and alanine racemase in 1 ml. The values of molar rotation $(\phi)_{365}$ are +1.08°, +7.44°, -0.80°, and -1.78° for L-alanine, L-lysine, L-serine, and L-methionine, respectively.

Reaction product was analyzed enantiometrically by the reversed-phase chromatography as described in "EXPERIMENTAL PROCEDURES" of CHAPTER I.

Protein was estimated by the Buiret method (Gonall et al., 1949), with bovine serum albumin as a standard. For most column fractions, the protein elution patterns were determined by absorption at 280 nm.

Purification of Alanine Racemase from B. stearothermophilus. All operations were performed at 0 - 5°C unless otherwise stated. Potassium phosphate buffer (pH 7.2) containing 10 M pyridoxal-P and 0.01% 2-mercaptoethanol was used as the standard buffer throughout the purification except where noted. All dialyses were performed with the seamless cellulose bags at 4°C for at least 6 h.

Step 1. Preparation of Crude Extract.----- The cells of B. stearothermophilus were grown in 50 liters of the medium, harvested by centrifugation at the end of the lag phase (about 18 h) and washed

with 0.85% NaCl. The washed cells (500 g, wet weight) were suspended in 500 ml of 10 mM buffer and disrupted by sonication at 4°C for 60 min with a 19 KHz Kaijo Denki osillator (Tokyo, Japan). The intact cells and debris were remobed by centrifugation.

Step 2. Ammonium Sulfate Precipitation.----- The supernatant solution (700 ml) was brought to 60% saturation with solid ammonium sulfate. After standing for 1 h the precipitate was collected by centrifugation, and dissolved in 300 ml of 10 mM buffer followed by dialysis against 30 liters of the same buffer.

Step 3. DEAE-Toyopearl Column Chromatography.----- The dialyzed solution was applied to a DEAE-Toyopearl 650M (Toyo Soda Co., Tokyo, Japan) column (10 X 40 cm) equilibrated with 10 mM buffer. After the column was washed with 2 liters of the buffer containing 20 mM KCl, the enzyme was eluted at a flow rate of 200 ml/h with the buffer containing 50 mM KCl. The active fractions were combined and concentrated with an Amicon 202 ultrafiltration unit.

Step 4. Phenyl-Sepharose CL-6B Column Chromatography.----- The enzyme solution was applied to a Phenyl-Sepharose column (2 X 10 cm) equilibrated with 1.0 M buffer. After successive washing with 0.8, 0.5, 0.25, 0.1 M phosphate buffer, the enzyme was eluted with the buffer at a flow rate of 200 ml/h. The active fractions were concentrated and dialyzed against 100 volumes of the buffer.

Isolation of Chromosomal DNA. The chromosomal DNA of B. stearothermophilus was isolated according to the modified method of Saito &

Miura (1963). The strain B. stearothermophilus IFO 12550 was cultivated in a 2-liter flask containing 500 ml of Medium A at 55 °C for 12 h. The cells harvested by centrifugation (wet weight, 5.0 g) were washed twice with 0.15 M NaCl containing 0.1 M EDTA (pH 8.0), and suspended in 40 ml of 0.1 M Tris-HCl buffer (pH 9.0) containing 0.1 M NaCl and 10 mM EDTA followed by treatment with 0.5 mg/ml lysozyme at 37°C for 20 min. Ten milliliters of 5% SDS in the Tris-HCl:NaCl:EDTA buffer were added gradually. The solution was incubated at 60°C for 20 min and treated with 1 mg/ml of pronase P at 37 °C for 4 h. The lysate was mixed with an equal volume of 80% phenol and gently shaken by hand for 30 min at room temperature. The resulting emulsion was centrifuged at 3,500 rpm for 10 min to separate the solution into two phases. The DNAs in the upper water layer were taken and precipitated with two volumes of cold ethanol under gentle mixing. The thread-like precipitate was rolled up with glass rods and washed successively with 70, 80 and 90% (v/v) ethanol with stirring. The DNAs were dissolved in 20 ml of 15 mM NaCl containing 15 mM sodium citrate (0.1 X SSC, SSC = saline sodium citrate), and 2 ml of 10 X SSC was then added to a final concentration of 1 X SSC. In order to remove the contaminated RNA, 50 μg/ml of RNase A was added, and the mixture was incubated at 37°C for 30 min. The digest was treated with 80% phenol and centrifuged as described above. The DNAs precipitated with cold ethanol were dissolved in 5 ml of 0.1 M Tris-HCl (pH 7.5) containing 0.1 M NaCl and 10 mM EDTA. After the solution was dialyzed against the same buffer

at 4°C overnight, the DNA solution was kept at 4°C until use.

Isolation of Plasmid DNA. Plasmid DNA was isolated by the modified method of Oka (1978). E. coli C600 cells carrying a plasmid were grown at 37°C in 100 ml of L-broth supplemented with appropriate antibiotics. When the cell density was reached to 3 X 10 /ml, chloramphenicol was added at a final concentration of 180 μg/ml. After incubation for 20 h, the cells were harvested by centrifugation and washed twice with 20 mM Tris-HCl buffer (pH 7.5) containing 0.14 M NaCl. The washed cells were suspended in 4 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 20 mM EDTA and 20% sucrose and treated with 0.5 mg/ml of lysozyme at 0°C. After 1 h, 1.4 ml of 5 M NaCl and then 0.2 ml of 20% SDS were added, and the lysation was completed by incubation at 37°C for 2 h. The lysate was left overnight at 4°C and centrifuged at 35,000 rpm for 30 min to remove chromosomal DNA and RNA. The supernatant solution was treated with 80% phenol. The resulting aqueous layer was concentrated by addition of cold isopropanol and treated with 100 μg/ml of RNase A at 37°C for 2 h. After the diggest was treated with 80% phenol, the DNA was collected by addition of cold ethanol and applied to a Bio-Gel A-5m (Bio Rad Laboratories Inc.) gel filtration column (1 X 30 cm). The plasmid DNA fractions monitored by absorption at 260 nm were precipitated with ethanol and dissolved in 10 mM Tris-HCl buffer (pH 7.5) containing 0.2 mM EDTA.

Digestion of DNA with Restriction Endonucleases. Reaction condi-

tions for digestion with restriction endonucleases were as follows:

EcoRI : 100 mM Tris-HCl buffer (pH 7.5), 7 mM $MgCl_2$, 50 mM NaCl, 7 mM 2-mercaptoethanol, and 0.01% BSA.

HindIII: 10 mM Tris-HCl buffer (pH 8.0), 7 mM $MgCl_2$, and 60 mM NaCl.

SalI : 10 mM Tris-HCl buffer (pH 7.5), 7 mM $MgCl_2$, 150 mM NaCl, 0.2 mM EDTA, 7 mM 2-mercaptoethanol, and 0.01% BSA.

PstI : 20 mM Tris-HCl buffer (pH 7.5), 10 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$, and 0.01% BSA.

Digestion with restriction endonucleases were carried out at 37°C, and the reaction was terminated by heating at 65°C for 5 min.
For digestion of plasmid DNA, enzymes were used at 2 u per μg of DNA, and incubations were carried out at 37°C for 16 h.

Ligation and Transformation. Ligations were carried out at 37°C for 16 h in 66 mM Tris-HCl buffer (pH 7.6) containing 6.6 mM MgCl , 10 mM dithiothreitol and 66 μM ATP. Transformation of E. coli was performed as described by Lederberg & Cohen (1974).

Construction of Vector Plasmid. Total genomic DNA (75 μg) from B. stearothermophilus was partially digested with SalI (50 u), and the resultant fragments were ligated into the SalI site of pBR322 (12 μg) by T4 DNA ligase (9 u). The ligated mixture was used directly for trasnsformation (Lederberg & Cohen, 1974). Transformants of E. coli C600 that contain these hybrid plasmids are resistant to ampicillin, but sensitive to tetracycline. To detect the L-alanine dehydrogenase-

producing colonies among these transformants, the replica printing method developed by Raetz (1975) was modified as follows: colonies of transformant grown on one of duplicate L-broth plates were transferred onto a Toyo No. 5C filter paper disc (diameter, 8cm). Colonies grown on the other plate were stored at 4°C until use. After treatment with lysozyme and EDTA as indicated (Raetz, 1975), filter paper discs in petri dishes were rapidly frozen in liquid N_2 followed by thawing in a water bath. This freezing and thawing was repeated twice. The filter paper dried in a water bath at 70°C for 20 min was then transferred to another petri dish containing 1.5 ml of a reaction mixture for L-alanine dehydrogenase assay. The mixture contained 50 mM glycine-KOH buffer (pH 10.5), 50 mM L-alanine, 0.625 mM NAD, 0.064 mM phenazine methosulfate and 0.24 mM nitro blue tetrazolium. The colonies producing L-alanine dehydrogenase appered as blue spots on the replica disc. Plasmid DNA was prepared from the L-alanine dehydrogenase-prodocing cells which were grown from the corresponding colony on the stored plate. Since the obtained plasmid pICR3 was rather large (11.0 kb) for the use as a cloning vector, the 3.2 kb HindIII fragment was deleted by digestion and religation of pICR3 (see Figure 2). Transformed *E. coli* containing the plasmid pICR301 thus obtained produced 2.37 units L-alanine dehydrogenase per mg of soluble protein. The plasmid pICR301 (7.8 kb) was used as a vector for cloning of the alanine racemase gene.

Gel Electrophoresis. Electrophoretic separation of DNAs was performed with 0.7% agarose gels in a Tris-borate-EDTA buffer system (Meyers et al., 1976) containing 0.5 μg of ethidium bromide per ml at a constant voltage. The gels were photographed on a Polaroid land pack film Type-655 under a long wavelength UV lamp with a Kodak No.23A red filter.

Spectrophotometry. Absorption spectra were taken with a Union Giken SM401 recording spectrophotometer or with a Shimadzu UV3000 recording spectrophotometer. Circular dichroism mesurement was done in a JASCO J-20 automatic recording spectropolarimeter.

Active Site Peptide Amino Acid Sequence Analysis. [^{3}H]$NaBH_4$ reduction of pure enzyme (5 mg), reductive alkylation, trypsin digestion and HPLC peptide purification were accomplished as described for the S. typhimurium dadB alanine racemase (Badet et al., 1984).

RESULTS

Partial Purification and Some Properties of Alanine Racemase from B. stearothermophilus.

(i) Partial Purification. The summary of the enzyme purification is presented in Table I. The alanine racemase was purified approximately 130 fold from the crude extract of B. stearothermophilus.

(ii) Identification of Reaction Product. Identification of

Table II Partial Purification of Alanine Racemase

Steps	Sp. Act. (U/Mg)	Fold (%)
1. Cell Free Extract	0.14	1
2. Ammonium Sulfate (0-60%)	0.34	2.4
3. DEAE-Toyopearl	2.88	21
4. Phenyl-Sepharose CL-6B	18.52	130

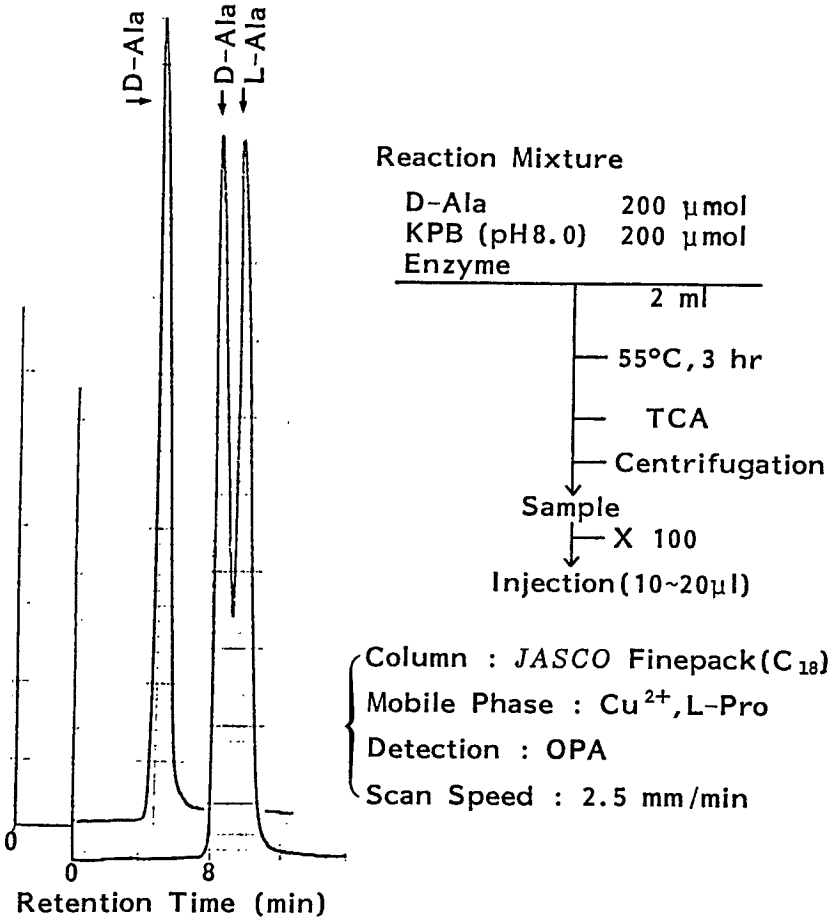


Figure 1: Identification of the reaction product by reversed-phase chromatography.

reaction product by the alanine racemase was performed with D-alanine as substrate by the reversed-phase chromatography. As shown in Figure 1, a new peak whose retention time was 10 min under the condition (the retention time of D-alanine = 8.5 min) and corresponded to that of L-alanine appeared on the chromatogram after 2 h incubation with the enzyme. The peak areas of D- and L-alanine are almost equal, indicating that the enzymatic racemization of D-alanine completed within 2 h.

(iii) Heat stability. When the partial purified enzyme in 0.1 M potassium phosphate buffer (pH 8.0) was heated at various temperatures for 10 min, the enzyme retained the following activities: 70 °C, 100%; 75°C, 95%; 80°C, 50%.

Construction of B. stearothermophilus Genomic Library. Total DNA was isolated from B. stearothermophilus IFO 12550 and digested with the restriction endonuclease HindIII to yield a population of fragments of average sizes of about 1 to 20 kilobases (kb). The fragments were then ligated into the HindIII site in the L-alanine dehydrogenase-encoded vector pICR301 and transformed into E. coli. A library of some 6,000 transformant (Amp^r, Tc^s) clones was obtained.

Alanine Racemase Screening of Cloned Library. The recombinant E. coli library was screened for the expression of B. stearothermophilus alanine racemase gene using an activity staining assay. Colonies were replicaplated (Figure 2), lysed in situ and probed with the L-alanine dehydrogenase assay described in "Experimental Procedures" except that D-alanine (50 mM) was used as a substrate instead of L-alanine. This

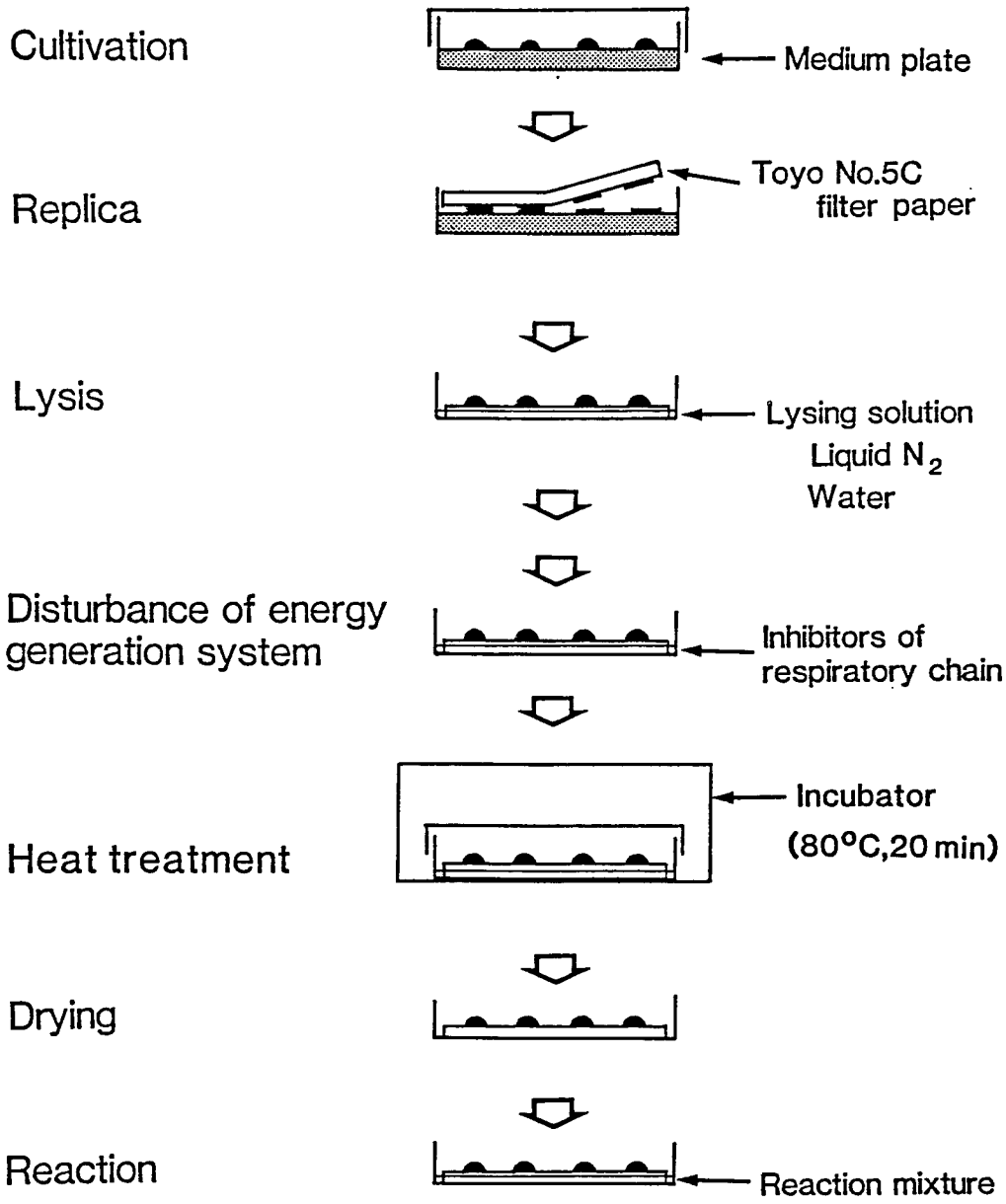
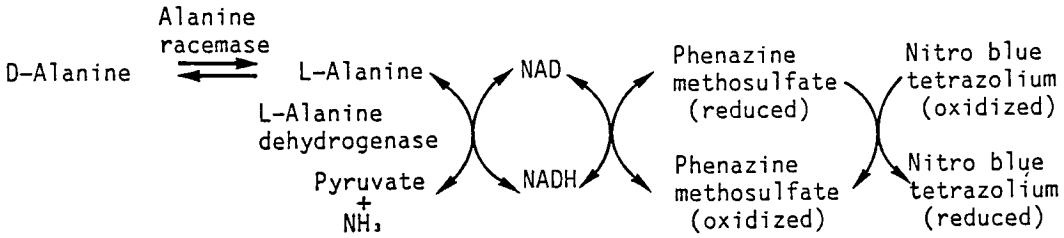


Figure 2: Procedure for screening of alanine racemase activity.

screening technique is based on the replica printing method developed by Raetz (1975) and recombinant E. coli cells producing alanine racemase are expected to show blue color of the reduced nitro blue tetrazolium as a result of electron transfer from phenazine methosulfate and NADH produced by L-alanine dehydrogenase in the recombinants:



In order to avoid non-specific color development caused by respiratory chain enzymes and alanine racemase from the host E. coli, the assay procedure involves heat treatment at 70°C for 20 min; both L-alanine dehydrogenase and alanine racemase from B. stearothermophilus are heat stable. Of approximately 6,000 Amp^rTc^S recombinants examined, only one colony turned blue on the replica plate. A plasmid band of aboaut 4.2 kb larger than the vector pICR301 was found in this positive clone upon agarose gel electrophoresis of a minipreparation of recombinant plasmid. The plasmid DNA was isolated from the clone, and designated pICR4 (12.0 kb, see Figure 3). The cell-free extract of the clone showed a high alanine racemase activity (see below).

Restriction Mapping and Subcloning of pICR4. Digestion of pICR4 with HindIII restriction endonuclease yielded a 4.2 kb fragment in addition to a 7.8 kb fragment corresponding to the vector plasmid

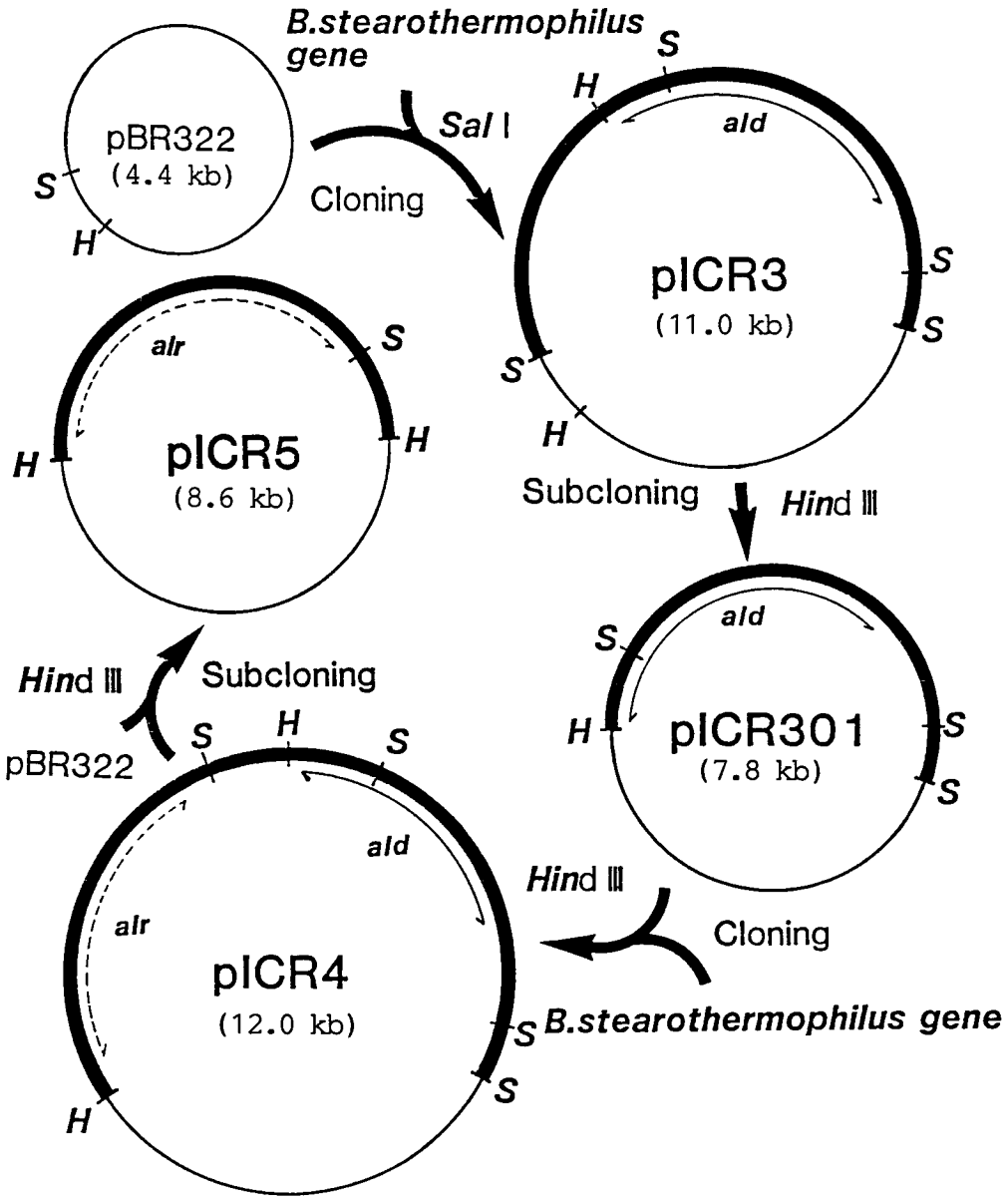
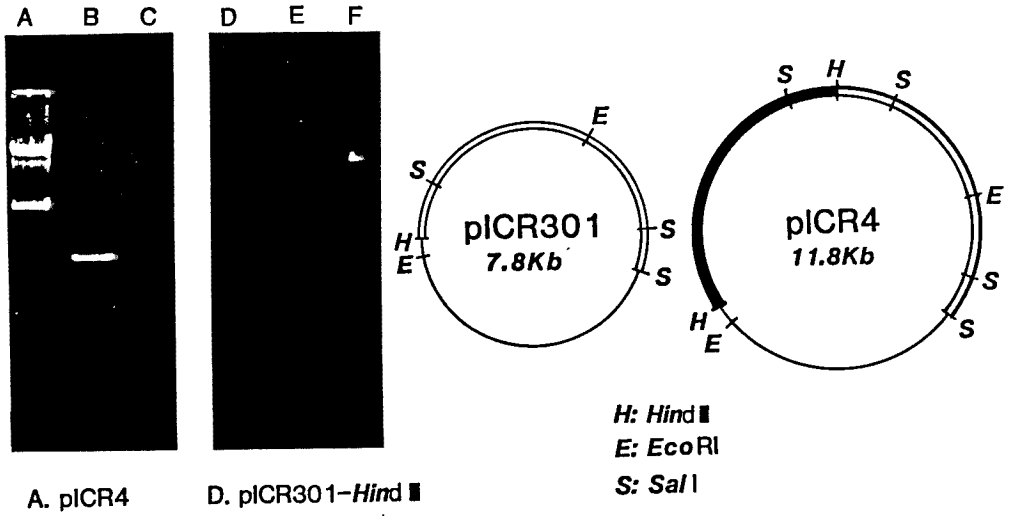


Figure 3: The construction scheme for and restriction maps of plasmids pICR4 and pICR5. The thin and heavy lines represent DNA fragments from pBR322 and *B. stearothermophilus* gene, respectively. Restriction endonuclease cleavage sites are *Hin*dIII *(H)*, and *Sal*I *(S)*. The DNA regions corresponding to the L-alanine dehydrogenase gene *(ald)* and the alanine racemase gene *(alr)* are represented arbitrarily. The numbers below the name of each plasmid are the sizes of the plasmids.

pICR301 (Figure 4). Thus, a 4.2 kb fragment derived from B. stearothermophilus genomic DNA was inserted into the HindIII site of pICR 301. By evaluating the electrophoretic patterns of pICR4 digested with single and double restriction endonucleases, the internal restriction map of the plasmid pICR4 DNA was obtained as shown in Figure 4.

The 4.2 kb HindIII fragment containing the alanine racemase gene was excised from pICR4 and recloned into the HindIII site of pBR322 to reduce the size of the plasmid by removing the L-alanine dehydrogenase gene and thereby to facilitate future analysis of the alanine racemase DNA sequence. One of $Amp^{r}Tc^{s}$ transformants obtained was found to express a high level of the alanine racemase activity (see Table II) and contain a plasmid, designated pICR5 which was mapped with restriction sites as shown in Figure 3. The transformant carrying pICR5 did not show the L-alanine dehydrogenase activity.

Figure 4: Agarose gel electrophoresis and restriction maps of pICR301 and pICR4.



Expression of Thermostable Alanine Racemase Gene in E. coli. The crude extract prepared from E. coli cells containing the plasmid pICR4, which carries the cloned B. stearothermophilus alanine racemase gene, as well as the alanine dehydrogenase gene, the ampicillin resistance gene and origin of replication from pBR322, showed about 30-fold higher level of alanine racemase activity than those from B. stearothermophilus cells and plasmidless E. coli C600 cells (Table II). Although the host E. coli C600 cells contain appreciable alanine racemase activity which is indistinguishable from the activity of the B. stearothermophilus enzyme by the assay method employed in this study, the 30-fold higher activity in E. coli-pICR4 suggests the high expression of the B. stearothermophilus alanine racemase gene. In addition, a marked increase in specific activity was observed when the cell extract from E. coli-pICR4 was heated at 70°C for 1 h. Such heat treatment precipitated most proteins including alanine racemase derived from E. coli, the thermostable enzyme activity being left in the supernatant. Thus, the specific activity of the E. coli C600-pICR4 extract was 33.5 U per mg of protein after the heat treatment, whereas no detectable activity was found in heat-treated cell extract of the host E. coli C600. This result indicates that the thermostable alanine racemase encoded by a gene from B. stearothermophilus is overproduced to about 0.3% of the soluble protein when carried on the plasmid pICR4 in E. coli. A high level of the gene expression was also found in E. coli C600 harboring the subcloned plasmid pICR5.

Table II. Alanine Racemase Activity in Crude Extract of *Bacillus stearothermophilus* and *E. coli* Clones

Strain	Specific Activity (units/mg)	
	Heat Treatment	
	before	after
B. stearothermophilus IFO12550	0.16	0.20
E. coli C600	0.15	0
E. coli C600 pICR4	4.81	33.5
E. coli C600 - pICR5	5.21	35.3

Cells (0.5 to 2 g) were disrupted by sonication as described under "Experimental Procedures". The enzyme activity was determined in the D to L direction at 100 mM D-alanine before and after heat treatment at 70°C for 1 h. The precipitated protein formed by the heat treatment was removed by centrifugation before assaying the enzyme.

Rapid Purification of Alanine Racemase from E. coli Carrying pICR4.

All operations were carried out at 0°C to 5°C. Potassium phosphate buffer (pH 7.2) containing 10 M pyridoxal-P and 0.01% 2-mercaptoethanol was used as the buffer throughout the purification except where noted.

Step 1. Preparation of Crude Extract.----- Cells of E. coli C600 carrying pICR4 harvested by centrifugation were washed twice with 0.85% NaCl. The washed cells (20 g, wet weight) were suspended in 100 ml of 10 mM buffer and disrupted by sonication at 0°C for 30 min with a 19 KHz Kaijo Denki oscillator. The intact cells and debris were removed by centrifugation.

Step 2. Heat Treatment.----- The supernatant solution was kept at 70°C for 1 h and was centrifuged after cooling in ice.

Step 3. Ammonium Sulfate Precipitation.----- The supernatant solution (80 ml) was brought to 60% saturation with solid ammonium sulfate. After standing for 1 h the precipitate was collected by centrifugation, and dissolved in 20 ml of 10 mM buffer followed by dialysis against 2 liters of the same buffer.

Step 4. DEAE-Toyopearl Column Chromatography.----- The dialyzed soluiton was applied to a DEAE-Toyopearl 650M column (1.5 X 10 cm) equilibrated with 10 mM buffer. After the column was washed with 100 ml of the buffer containing 20 mM KCl, the enzyme was eluted at a flow rate of 150 ml/h with the buffer containing 50 mM KCl. The active fractions were combined and concentrated with an Amicon 202 ultrafiltration unit.

Step 5. Sephadex G-150 Column Chromatography.----- The enzyme solution was applied to a column (2 X 100 cm) of Sephadex G-150 equilibrated with 10 mM buffer and eluted at a flow rate of 10 ml/h. The active fractions were pooled and concentrated by ultrafiltration.

Table III. Purification of Alanine Racemase from *E. coli* C600 Carrying pICR4

Steps	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)
1. Crude Extract	1340	6300	4.7	100
2. Heat treatment	230	6070	26.4	96
3. Ammonium Sulfate	120	5660	47.2	90
4. DEAE-Toyopearl	5.0	5280	1055	84
5. Sephadex G-150	2.5	4030	1610	64

Starting with 20 g cells of E. coli C600-pICR4, the B. stearothermophilus alanine racemase was purified by five steps including two chromatographic procedures (Table III). The heat treatment of cell-free extract was found to be very effective for the enzyme purification as described above: five to seven fold purification was achieved without loss of total enzymatic activity. Ion exchange chromatography with DEAE-Toyopearl was essential in the following procedure, giving typically a twenty fold purification. The enzyme purified 340-fold with a 64% final yield appeared to be homogeneous by the criteria of polyacrylamide gel electrophoresis and analytical ultracentrifugation.

Immunochemical Analysis. The antiserum against thermostable alanine racemase purified from E. coli-pICR4 was used to investigate its immunochemical identity with the B. stearothermophilus enzyme. The antiserum reacted with the partially purified alanine racemase from B. stearothermophilus as well as with the purified enzyme from E. coli-pICR4, crude extract of E. coli-pICR4, and crude extract of E. coli-pICR5, producing a single line of precipitation with complete fusion (Figure 5). No cross reaction against the antiserum was observed with the cell-free extract of E. coli C600 and E. coli containing the vector plasmid pICR301. The result indicates that the alanine racemase produced by E. coli carrying pICR4 or pICR5 is immunochemically identical with the B. stearothermophilus enzyme, and therefore confirms that the B. stearothermophilus alanine racemase gene is expressed in E. coli.

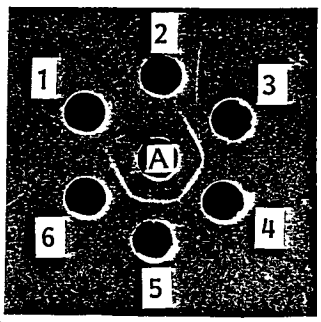


Figure 5: Ouchterlony double-diffusion analysis of alanine racemase. The center well contains the antiserum against alanine racemase purified from *E. coli* C600 containing pICR4. Wells 1 to 4 each contain 50 µl of crude extracts of *E. coli* C600 carrying various plasmids; 1, no plasmid; 2, pICR 301; 3, pICR4; and 4, pICR5. Wells 5 and 6 contain, respectively, homogeneous alanine racemase purified from *E. coli* C600-pICR4 and partially purified alanine racemase of *B. stearothermophilus*.

Physical and Kinetic Characterization. The sedimentation coefficient (S^0_{20w}) of the enzyme was calculated to be 5.4 S. Assuming a partial specific volume of 0.74, the molecular weight of 78,000±2,000 was obtained by the sedimentation equilibrium method. The subunit structure was examined by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). The SDS-treated enzyme was subjected to electrophoresis in the presence of 0.1% SDS and migrated as a single protein band (Figure 6). The molecular weight of the band was estimated to be about 39,000 based on its mobility relative to those of standard calibration proteins (Figure 6). These results show that the enzyme is composed of two subunits identical in molecular weight.

The enzyme showed maximum reactivity at around 50°C with a Vmax value of 1800 u/mg (D- to L-alanine). The temperature dependence of Vmax was analyzed by Arrhenius plots, and the activation energy Ea was 1.8 kcal/mol with calculated values of ΔH^*, ΔG^* and ΔS^* = 1.18

kcal/mol, 18.46 kcal/mol and -55.7 cal/mol deg., respectively. At 37 °C in 100 mM 2-(N-cyclohexylamino)ethanesulfonic acid buffer (pH 9.0), the Km for D-alanine is 2.67 ± 0.2 mM and Vmax for racemization (D to L) is 1400 u/mg. In the L-alanine to D-alanine direction, Km of 4.25 ± 0.2 mM and Vmax of 2550 u/mg were obtained by the D-amino acid oxidase coupled assay (Badet & Walsh, 1985). When these values were used, the calculated Keq for alanine racemization was 1.14, in good agreement with the theoretical value (1.0) for the chemically symmetric reaction L-alanine⇌D-alanine (Briggs & Haldane, 1925). Alanine is the exclusive substrate for the enzyme; L-serine, L-methionine, L-

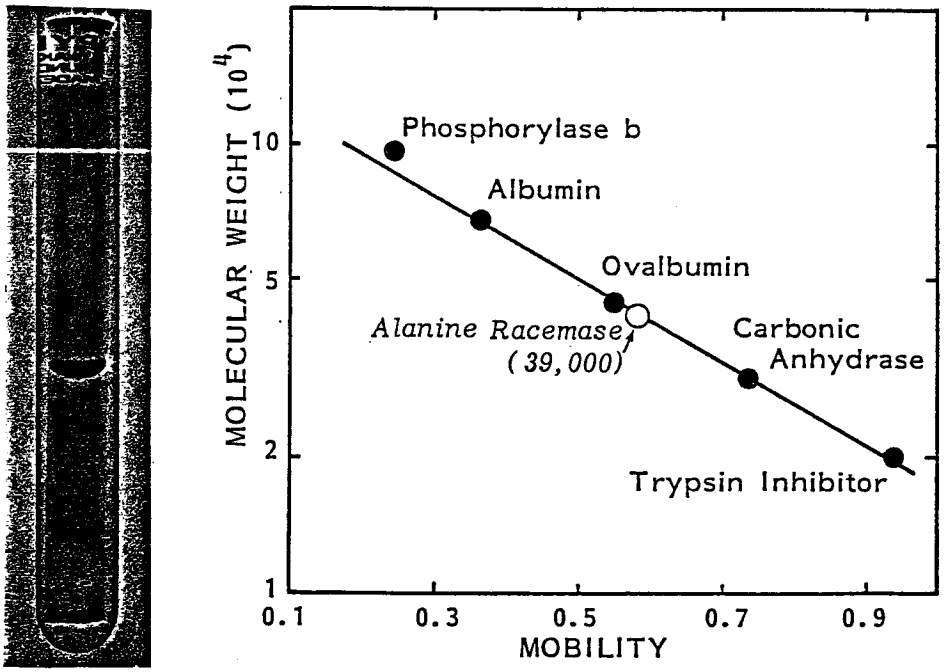


Figure 6: Determination of molecular weight of alanine racemase subunit by SDS gel electrophoresis. After the purified enzyme (20 µg) was treated with 1.0% SDS in 10 mM sodium phosphate buffer (pH 7.2) containing 1.0% 2-mercaptoethanol and 25% glycerol at 37°C overnight, the treated enzyme preparation was subjected to electrophoresis in the presence of 0.1% SDS with 10% polyacrylamide gels in the Tris-glycine buffer system (Laemmli, 1970).

lysine, L-valine, L-homoserine and L-α-aminobutyrate (100 mM) were not racemized when examined by the polarimetric assay.

Enzymatic activity was found quite stable upon heat treatment at 70°C for 80 min in O.1 M potassium phosphate buffer (pH 7.2)(Figure 7).

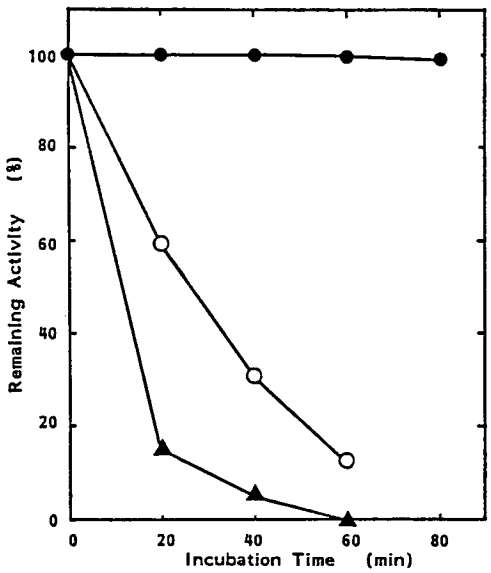


Figure 7: Heat stability of alanine racemase. The enzyme was subjected to the heat treatment with 0.1 M potassium phosphate buffer (pH 8.0). The remaining activity was determined under the standard conditions. (●), heated at 70°C; (○), heated at 80°C; (▲), heated at 90°C.

Amino Acid Composition. The amino acid composition of the purified enzyme is given in Table IV. The predominant residues of the enzyme protein were glutamic acid, aspartic acid, arginine, alanine, and leucine. No other striking feature for a thermostable enzyme was observed except that the enzyme contained only two half-cystine per monomeric unit. The total of integral numbers of each amino acid residues gave a calculated molecular weight of about 38,000 for the polypeptide chain. The amino acid composition is quite similar to those of amino acid racemases from gram negative bacteria, Salmonella typhimurium (Badet et al., 1984) and Pseudomonas putida (= Ps. stria-

ta) (Roise et al., 1984). Thus, a statistical analysis of the amino acid compositions in a mole percent basis by the method of Harris et al. (1969) yielded low deviation functions($D = (\Sigma (X_{1i} - X_{2i})^2)^{\frac{1}{2}}$) between the B. stearothermophilus alanine racemase and the S. typhimurium dadB alanine racemase (D = 0.056) and between the B. stearothermophilus alanine racemase and the A. caviae amino acid racemase (D = 0.079).

Table IV. Amino Acid Composition of Alanine Racemase

Amino Acid	Number of Residues (mol/mol of Enzyme)			Number of Proposed Amino Acid Residues
	24 h	48 h	72 h	
Aspartic acid	24.5	27.9	28.2	28
Threonine	13.8	17.9	18.8	19
Serine	13.4	16.1	15.9	16
Glutamic acid	25.6	29.0	29.5	29
Proline	18.8	19.8	19.3	19
Glycine	20.0	22.0	21.8	22
Alanine	30.3	32.8	32.6	33
Half-cystine	0.9	2.3	2.4	2
Valine	15.3	20.4	22.0	22
Methionine	6.9	7.4	7.5	7
Isoleucine	12.4	15.8	17.2	17
Leucine	26.5	29.9	30.5	30
Tyrosine	8.2	10.6	9.9	10
Phenylalnine	10.5	12.6	13.0	13
Lysine	8.3	10.8	11.3	11
Histidine	10.6	12.0	12.6	12
Arginine	20.9	24.2	24.9	24
Tryptophan				14
Total Number of Residues				328

The protein was hydrolyzed at 110°C for 24, 48, and 72 h. The results were averaged and the integral number of the amino acid is presented. Details for the experiment are given in the text.

Cofactor Analysis and Spectrometric Properties. Althouth some earlier studies showed that the alanine racemases from Staphylococcus aureus (Strominger et al., 1960), Pseudomonas sp. (Rose & Strominger, 1966), and Escherichia coli (Lambert & Neuhaus, 1972) did not require pyridoxal-P as a cofactor, recent reports have clearly indicated its presence in homogeneous alanine racemase preparations (Badet et al., Roise et al., Wasserman et al., 1984; Badet & Walsh, 1985). I also determined the pyridoxal-P content in the purified B. stearothermophilus alanine racemase by the phenylhydrazine method (Wada & Snell, 1961) and the MBTH method (Soda et al., 1969) and found that two mol of pyridoxal-P was bound per mol of enzyme molecule (dimer) by use of a molecular weight of 78,000. This shows that one mol of pyridoxal-P is bound to one subunit of the enzyme.

The purified enzyme exhibits absorption maximum at 420 nm in the visible region (Figure 8A), showing a typical Schiff base formed between the enzyme protein and pyridoxal-P. The extinction coefficient at 420 nm was calculated to be 16,900 $M^{-1}cm^{-1}$ with the A_{280}/A_{420} ratio of about 5.6. No appreciable spectral shifts occurred by varying the pH (6.0 to 9.0). Reduction of the enzyme with sodium borohydride by the dialysis method of Matsuo & Greenberg (1959) caused a disappearance of the 420-nm peak and an increase in absorbance at about 330 nm (Curve C in Figure 8A) with a concomitant loss of the enzyme activity. The holoenzyme was converted to the inactive apoenzyme (Curve B in Figure 8A) by treatment with 10 mM NH_2OH (pH 8.0) and then dialysis against

10 mM potassium phosphate buffer (pH 8.0) containing 0.1% 2-mercaptoethanol. Incubation of the apoenzyme with various concentrations of pyridoxal-P at 30°C for 2 h followed by measurement of the enzyme activity gave an apparent Michaelis constant for pyridoxal-P in the range of 1.5 - 2.0 M. Circular dichroic spectrum of the enzyme showed a negative peak at 420 nm (Figure 8B), corresponding to the absorption peak.

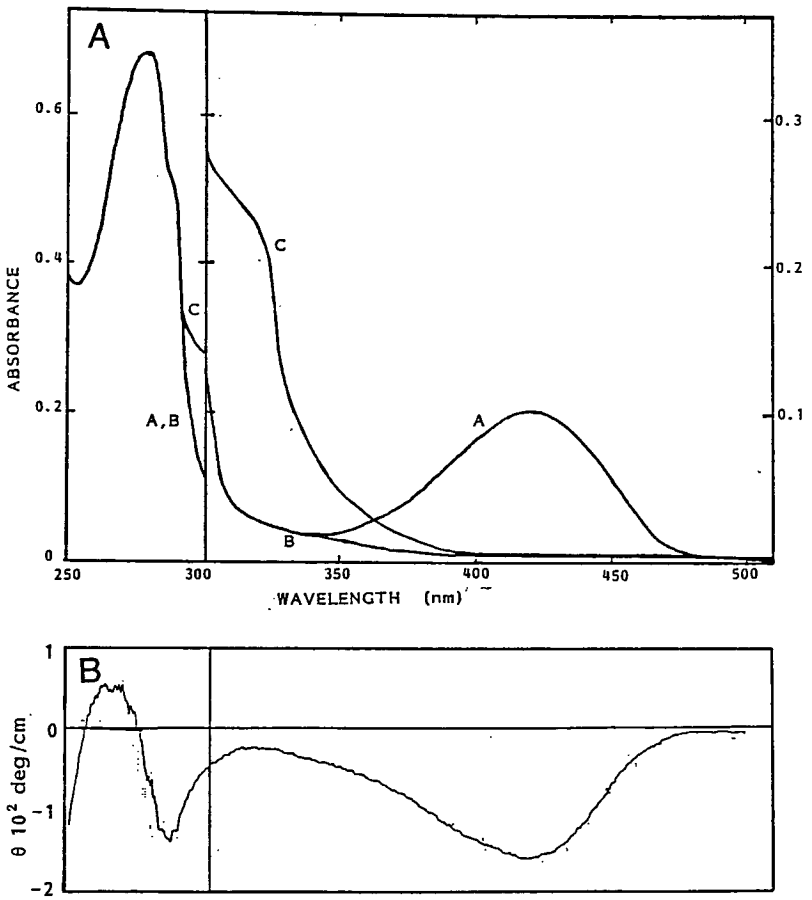


Figure 8: Absorption (A) and circular dichroic (B) spectra of alanine racemase. A, the absorption spectra wewre taken in 10 mM potassium phosphate buffer (pH 8.0 at the enzyme concentration of 0.6 mg/ml; Curve A, holoenzyme; Curve B, apoenzyme; Curve C, $NaBH_4$-reduced enzyme. B, the circular dichroic spectrum was taken in 10 mM potassium phosphate buffer (pH 8.0).

A negative dichroic peak caused by bound cofactor has also been observed for the broad substrate specificity amino acid racemase of A. caviae (see Figure 3B in CHAPTER I) and Ps. putida (K. Tanizawa & K. Soda, unpublished results), and suggests that optical properties around the active site of these racemases are similar with each other.

Active Site Peptide Structure of B. stearothermophilus Alanine Racemase. The partial sequence of the active site tryptic peptide from the enzyme was determined and compared with the sequences of three other racemases (Badet & Walsh, 1984; Roise et al., 1984; Esaki & Walsh, unpublished results). The usual procedure of B[^{3}H]$^-$ reduction of native enzyme (5 mg) to generate [^{3}H]-PNP-ϵNH$_2$-Lys-enzyme was followed by reductive alkylation and tryptic digestion of the reduced enzyme, HPLC isolation of radiolabeled peptides and automated Edman degradation for sequence determination (Badet et al., 1984). Eight residues around the [^{3}H]-PNP-Lys adduct could be unambiguously determined and are shown in Table V in comparison with the broad substrate specificity amino acid racemase of Ps. putida and the dadB and dal gene-encoded Salmonella racemases. These results indicate the marked homology of the active site sequence in the gram negative and gram positive racemases.

Table V. Sequence Homology of the Two Racemase Active Site Peptides

Amino Acid Racemase	
Pseudomonas putida	NH_2-Ala-Val-Leu-Lys*-Ala-Asp-Ala-Tyr-COOH
Alanine Racemase	
Salmonella typhimurium (dadB)	-Ser-Val-Val-Lys*-Ala-Asn-Ala-Tyr-
(dal)	-Ala-Val-Val-Lys*-Ala-Asn-Ala-Tyr-
Bacillus stearothermophilus	-Ala-Val-Val-Lys*-Ala- X -Ala-Tyr-

* N^6-Phosphopyridoxyl-L-lysine

DISCUSSION

A B. stearothermophilus gene coding for alanine racemase has been cloned and expressed in E. coli C600 with the vector plasmid pICR301 carrying the thermostable L-alanine dehydrogenase gene. Clones were selected for the ability to reduce nitro blue tetrazolium with NADH which was generated inside E. coli cells by the action of L-alanine dehydrogenase on NAD and L-alanine produced from D-alanine by alanine racemase. Although the respiratory chain enzymes and alanine racemase contained in the host E. coli cells caused undesirable color development of the reduced nitro blue tetrazolium in the replica printing assay, the heat treatment of the printed colonies was quite effective to minimize the back ground color, destroying most of thermolabile enzyme activities in E. coli. The amount of racemase in the isolated recombinant cells is estimated to be about 0.3% of the soluble cellular protein (based on the specific activity in the E. coli-pICR4 supernatant as compared to the specific activity of the purified racemase), which is elevated 30-fold over the wild-type level. The alanine racemase purified to homogeneity from E. coli-pICR4 was confirmed to be immunochemically identical with the enzyme of B. stearothermophilus (see Figure 5).

Since alanine racemases generally function constitutively in the cell wall biosynthesis and their intracellular content is very low, attempts to purify the enzymes to a homogeneous state from wild bacte-

rial strains have been limited or unsuccessful (Diven et al., 1964; Rose & Strominger, 1966; Yonaha et al., 1975). Badet & Walsh (1985) had to purify the Streptococcus faecalis enzyme 25,000 to 30,000 fold to homogeneity, little protein being available for extensive work. In this respect, the cloning of a thermostable enzyme gene in E. coli is also of great advantage for purification of the expressed gene product. As demonstrated in this paper, the heat treatment of cell free extracts raised the specific activity of alanine racemase 5-7 fold without loss of total activity. Thus, subsequent purification procedures were greatly facilitated. Gene cloning of such thermostable enzymes and rapid purification by the heat treatment of E. coli cell extract have been successfully employed for 3-isopropylmalate dehydrogenase from an extreme thermophile Thermus thermophilus (Nagahari et al., 1980; Tanaka et al., 1981) and L-leucine dehydrogenase from a moderate thermophile Bacillus stearothermophilus (S. Nagata & K. Soda, unpublished results). These results would provide a good system for purification of various thermostable enzymes of thermophilic bacteria.

The homogeneous alanine racemase of B. stearothermophilus isolated from the overproducing E. coli recombinant cells is now available in quantity and allows us to compare its enzymological properties with those of other well-characterized amino acid racemases. The dimeric structure with a molecular weight of about 78,000 and some other physicochemical properties (e.g. amino acid composition, absorption and circular dichroism spectra) are very similar to those of the

broad specificity amino acid racemase from Ps. putida (Soda & Osumi, 1969). In contrast, the alanine racemase encoded by the Salmonella typhimurium dadB gene (Wasserman et al., 1984) and the enzyme of Streptococcus faecalis (Badet & Walsh, 1985) are believed to be a non-spherical monomer with a molecular weight of around 40,000 in their native state. Kinetic parameters for alanine racemization by the B. stearothermophilus enzyme are similar in magnitude to those of the enzymes from S. typhimurium (Wasserman et al., 1984), S. faecalis (Badet & Walsh, 1985) and Ps. putida (Roise et al., 1984), and fit well to the Haldene relationship (Briggs & Haldane, 1925) giving Keq = 1.14 in agreement with the predicted value of 1.0. The extensive homology of the active site phosphopyridoxyl peptide among the B. stearothermophilus enzyme, the S. typhimurium enzyme (Badet et al., 1984), and the Ps. putida enzyme (Roise et al., 1984) strongly suggests that these racemases have been evolved from a common progeniter.

Recent studies with a newly developed alanine racemase inhibitor, (1-aminoethyl)phosphonic acid (Ala-P), have revealed that the inhibition by this compound is time-dependent and irreversible for the enzymes of gram positive bacteria, while reversible for the enzymes of gram negative bacteria (Atherton et al., 1980; Badet & Walsh, 1985). Although the mode of irreversible inactivation of alanine racemases from gram positive bacteria has been characterized in detail (Badet & Walsh, 1985; Badet et al., 1985), the molecular basis of the distinct action of Ala-P on enzymes from two types of bacteria has been unknown

and will require three dimensional structures of these racemases to be clarified. The thermal stability of the B. stearothermophilus racemase would thus make it a good candidate for high-resolution X-ray analysis. DNA sequence to provide the encoded primary structure of the B. stearothermophilus racemase is underway.

SUMMARY

The alanine racemase (EC 5.1.1.1) gene of a thermophilic bacterium, Bacillus stearothermophilus was cloned and expressed in Escherichia coli C600 with a vector plasmid pICR301 which was constracted from pBR322 and the L-alanine dehydrogenase gene derived from B. stearothermophilus. A coupled assay method with L-alanine dehydrogenase and tetrazolium salts was used to detect visually the alanine racemase activity in the clones. Alanine racemase overproduced in a clone carrying the plasmid pICR4 of 12-kb DNA was purified from cell extracts about 340-fold to homogeneity by five steps involving heat treatment. The enzyme was confirmed to originate from B. stearothermophilus by an immunochemical cross reaction with the enzyme of B. stearothemophilus. The purified enzyme has a molecular weight of about 78,000 and consists of two identical subunits of a molecular weight of 39,000. At the optimum temperature (50°C) the enzyme has a specific activity of 1800 units/mg (Vmax, D- to L-alanine). Resolution and reconstitution experiments together with the absorption spectrum of the enzyme clearly indicate that alanine racemase of B. stearothermophilus is a pyridoxal-P enzyme.

CONCLUSION

In this study, the author dealt with two amino acid racemases.

In CHAPTER I, the localization in the cells, the purification and some properties of the broad substrate specificity amino acid racemase are described. The amino acid racemase has been purified to homogeneity from cell-free extract of the isolated mesophile Aeromonas caviae (= Aeromonas punctata subsp. caviae). The enzyme consists of two identical polypeptide chain (molecular weight 40,000) and requires pyridoxal-P as a cofactor (Km = 2.6 μM). The enzyme exhibits absorption maxima at 280 nm and 420 nm, and the enzyme has been found to contain two mol of pyridoxal-P per mol of enzyme. Enzymological and physicochemical properties of the enzyme were compared with those of the two enzymes from Ps. putida and Ps. taetrolens. Although the amino acid racemase of A. caviae is immunochemically distinct from the enzyme of Ps. putida, these two broad substrate specificity amino acid racemases are very similar in many points. Furthermore, these enzyme show similar reactivity against L-MTCC. This result suggests the similarity of the active site structure.

In CHAPTER II, the molecular cloning and expression of the alanine racemase gene of thermophile B. stearothermophilus and the rapid purification of this enzyme from the clone to compare its properties with those of other amino acid racemases, particularly the broad substrate specificity amino acid racemase described in CHAPTER I.

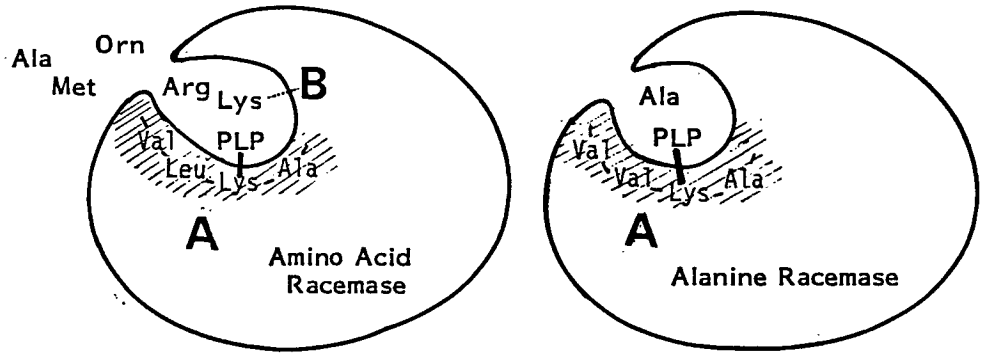
An alanine racemase gene of B. stearothermophilus has been cloned and expressed in E. coli C600 with the vector plasmid pICR301 carrying the thermostable L-alanine dehydrogenase gene. Alanine racemase overproduced in a clone carrying the plasmid pICR4 was rapidly purified from cell extracts about 340-fold to homogeneity by five steps involving heat treatment. The purified enzyme has a molecular weight of about 78,000 and consists of two identical subunits of a molecular weight of 39,000. This enzyme also contains two moles of pyridoxal-P per mole of enzyme and exhibits similar absorption and CD spectra.

The above-mentioned results summarized in TABLE. Although the broad substrate specificity amino acid racemase is clearly distinct from the alanine racemase in its substrate specificity, these enzymes have similar physicochemical properties. These enzymes are

TABLE Properties of Alanine Racemase and Amino Acid Racemase

Properties	Alanine Racemase of		Amino Acid Racemase of	
	B.stearo.	*S.typhimurium*	*A.punctata*	*Ps.putida*
Mol. Wt.	: 78,000	50,000 (?)	80,000	84,000
S_{20w}	: 5.4 S	—	5.5 S	5.6 S
λ_{max}	: 280 nm 420 nm	280 nm 420 nm	280 nm 420 nm	280 nm 420 nm
CD (+,-)	: 420 nm (-)	—	420 nm (-)	420 nm (-)
$E^{1\%}_{280}$	: 11.9	—	10.9	8.5
Subunit (mol. Wt.)	: 2 identical (39,000)	Monomer(?) (40,000)	2 identical (40,000)	2 identical (42,000)
Cofactor	: PLP 2 mol/mol Enz	PLP 1 mol/mol Enz	PLP 2 mol/mol Enz	PLP 2 mol/mol Enz
Optimum Temp.	: 50℃	—	60℃	45℃
Optimum pH	: Ala 8.0 - 9.0	—	Lys 7.5 - 9.0	Lys 7.5 - 9.0
Substrate	: Ala	Ala	Lys,Orn,Arg Gln,Met,Asn, Ala,Ser,etc.	Lys,Orn,Arg Gln,Met,Asn Ala,Ser,etc.
Km	: PLP 1.1μM D-Ala 2.7mM	— D-Ala 2.0 mM	PLP 2.6μM D-Lys 0.2 mM	PLP 24μM D-Lys 0.1 mM

composed of two identical subunits (molecular weight, approximately 40,000), and contains one mole of pyridoxal-P per mol of subunit. These results and homology of active site structure (see Table V in CHAPTER II) strongly suggest that these racemases have been evolved molecularly from a common progenitor enzyme. The distinction of substrate specificity between these enzymes is probably due to slight difference in the protein structure near the active site as shown in the figure below. In addition to the common active site (Site-A, pyridoxyl peptide region), the broad substrate specificity amino acid racemase has another binding site (Site-B, the basic amino acid binding region).



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